VOL. XLII JUNE, 1920 No. 2

THE JOURNAL OF BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

OFFICIAL ORGAN OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

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PUBLISHED MONTHLY
AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
FOR THE JOURNAL OF BIOLOGICAL CHEMISTRY, INC.

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Entered as second-class matter August 1, 1911, at the Post Office at Baltimore, Md., under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorized on June 29, 1918.

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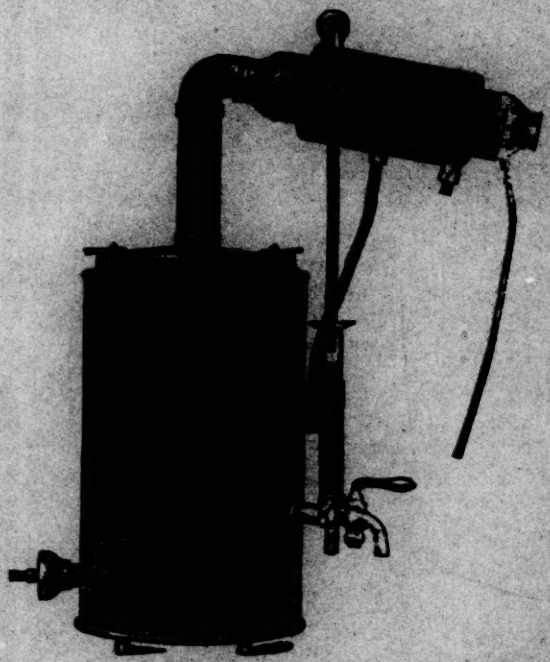

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HEMATO-RESPIRATORY FUNCTIONS.*

II. THE HENDERSON-MORRISS METHOD FOR DETERMINING THE CARBON DIOXIDE IN PLASMA AND IN WHOLE BLOOD.

By HOWARD W. HAGGARD.

(From the Physiological Laboratory, Yale Medical School, New Haven.)

(Received for publication, April 19, 1920.)

A subnormal CO_2-combining power in the plasma of the blood is now regarded as an index of acidosis. The values obtained from whole blood have to a large extent been neglected. For reasons which will be stated in a succeeding paper it is often important and sometimes essential to use the whole blood. Whole blood has also the advantage of economy; to get a unit of plasma one needs two or three units of blood.

The use of the CO_2-combining power as an index of the alkali and thus indirectly of the C_H of the blood, was introduced by Hasselbalch and developed by Van Slyke. Hasselbalch regards determinations on plasma as much less significant than on whole blood. The former may at times be absolutely misleading. After careful comparison of whole blood and plasma Hasselbalch and Warburg (1) concluded that the CO_2-binding power of a serum rises and falls with the CO_2 tension of the blood from which it is separated. It is not possible to draw from the CO_2 of the serum any exact conclusion whatever regarding the blood reaction, unless the CO_2 tension of the blood, when the serum was separated, has been taken into account. Experience in this laboratory fully confirms this statement. In fact, although Van Slyke's statement to a similar effect has been generally overlooked, the truth of this was emphasized by him also. Carbonating the plasma after separation is comparatively unimportant.

* This work was done under the War Gas Investigations of the Bureau of Mines and the Chemical Warfare Service, U. S. Army. The writer is indebted to H. H. Beatty, S. R. Detwiler, and W. H. Taliaferro for valuable collaboration.

In a paper from this laboratory (2) published a little over 2 years ago a simple method was described for determining the CO_2 content and capacity, or combining power, of the whole blood. Since that time this method has been in continuous use on an extensive scale in this laboratory, and we have now acquired an experience which allows us to judge of its value.

The Van Slyke and Cullen (3) method for determining the CO_2 capacity, or alkaline reserve, of plasma has been used by us interchangeably with this method. For plasma the two methods are of about equal rapidity, simplicity, and accuracy. For the determination of CO_2 in whole blood the necessity of cleaning the Van Slyke apparatus after each analysis is troublesome and time-consuming. The Henderson-Morriss method offers no more difficulties with whole blood than with plasma. The gas burette serves also to analyze the equilibrating air—an essential for exact work.

The paper describing the Henderson-Morriss method was of a preliminary character. It was therefore suggested to us by Henderson that we reconsider in detail the calculation of results by this method and report a series of carefully performed comparative determinations with the Van Slyke procedure; also, that we rectify a typographical error in the barometric correction.[1]

In the Henderson-Morriss method 1 cc. of blood is laked with dilute ammonia and then acidulated with tartaric acid, 20 per cent, in a glass tube of simple form which contains when stoppered exactly 3.5 cc. of acid-blood mixture and approximately 24 cc. of air. The tube is then rolled so that the fluid spreads in a thin film allowing free diffusion between fluid and air. 3 minutes are sufficient to bring the tension of the CO_2 in the air and fluid to equilibrium. The tube is then connected with the gas burette. The lower end is in a pan of water. The stopper is withdrawn under water. The total amount of gas (air plus the CO_2 liberated by acid) is drawn into the burette, water following up the tube from the pan. The end of the tube is lifted sufficiently to admit a bubble of air which is then used to wash the last portion of the air in the tube into the burette. The burette is then read, the gas passed five times into the absorbing bottle containing 10 per cent NaOH, and the burette is again read. The decrease of volume observed, when subjected to the corrections described below, indicates the volumes per cent of CO_2 contained in the blood. With a burette of this type, time must be allowed for the fluid, acidulated water, to drain fully

[1] Henderson and Morriss (2), p. 224.

before each reading. Quicker draining without loss of accuracy in any other respect is afforded if water plus 10 per cent alcohol and a few drops of HCl are used in the burette. It is well also to add a few drops of litmus, phenolphthalein, or other indicator to this fluid so as to warn one in case any of the alkali is drawn over by mistake.

Our practice is to measure the blood in a Mohr pipette which has been recalibrated with mercury. The outside of the pipette should be carefully wiped free from blood before the contents are delivered into the diffusion tube. The commonest source of error is in a "long" measurement with the pipette.

We have found it advisable to substitute a 2-way stop-cock for the pinch-clamp between the burette and the absorption bottle as originally figured.

A correction is made for the CO_2 liberated, on acidulation, from the small traces of carbonate in the ammonia solution. This covers also the atmospheric CO_2 in the air in the diffusion tube. The correction consists in subtracting from the observed volume of CO_2 as read on the burette the value obtained from a blank determination in which distilled water replaces the blood.

The observed reduction of volume in the gas burette, *i.e.* the CO_2 absorbed by the alkali, is reduced to the volume of the dry gas at 760 mm. by the formula

$$V_1 = \left(\frac{B - p}{760}\right)(V - \text{Cor.})$$

$(V - \text{Cor.})$ = Volume of CO_2 after correction for carbonate in reagents.
p = Vapor tension of water vapor at temperature of analysis.
B = Barometer reading.
V_1 = Volume of dry gas at 760 mm

To the volume of dry gas is added the CO_2 retained in solution in the fluid in the diffusion tube. This is estimated by the formula

$$V_2 = V_1 + V_1\left(\frac{3.5}{24} \times c\right)$$

V_2 = Volume of CO_2 in the gas analyzed + that remaining in solution in the acidulated blood.
V_1 = As above.
3.5 = Fluid contained in diffusion tube.
24 = Air " " " "
c = Solubility coefficient of CO_2 in water at temperature of analysis.

The correctness of this value for c as applied to the acid-blood mixture will be discussed later.

The value of V_2 at the temperature of analysis is brought to 0° by the formula

$$V_3 = V_2\left(\frac{273}{273+t}\right)$$

V_3 = Volume at 0°.
V_2 = As above.
t = Temperature of analysis.

The volume of CO_2 in cc. at 0° (*i.e.*, V_3) multiplied by 100 gives the volumes per cent of CO_2 in the blood.

The Solubility Coefficient of CO_2 in the Acid-Blood Mixture.

The solubility coefficient of CO_2 in the acidulated blood is so nearly identical with that for CO_2 in water that the established values for the latter may be used in determining the amount of CO_2 left in solution after the equilibrating air has been analyzed. This was determined by displacing the gas with mercury instead of water (so as not to expose or dilute the acidulated blood), then drawing air freed from CO_2 into the diffusion tube,

TABLE I.

CO_2 Retained in Acid-Blood Mixture.

Barometer 758 mm.

Blood samples.	Temperature.	CO_2 in equilibration.					
		No. 1.	No. 2.	No. 3.	No. 4.	Sum of Nos. 2, 3, and 4.	Theoretical for distilled water.
	°C.	cc.	cc.	cc.	cc.	cc.	cc.
A	21	0.34	0.040	0.005	0.000	0.045	0.044
B	21	0.53	0.065	0.010	0.005	0.080	0.069
C	21	0.47	0.065	0.010	0.000	0.075	0.061
D	18	0.44	0.060	0.005	0.005	0.070	0.062
E	18	0.38	0.050	0.005	0.005	0.060	0.054

and again equilibrating. From the analyses of the gas thus obtained in a series of equilibrations amounts of CO_2 were found by analysis approaching zero as a limit. The sum of these added to the CO_2 of the initial equilibration gives the total volume of CO_2 in the blood. For these analyses a gas burette more finely graduated than that generally employed was used. The results of such determinations are given in Table I.

From these figures we conclude that the CO_2 retained in solution in the fluid in the diffusion tube can be calculated by the employment of the solubility coefficient of water to within 1 volume per cent.

The various corrections and calculations just described when applied to this method so neutralize each other that for routine work they are unessential. *The volume of CO_2 absorbed from the air in the burette (minus only the correction for CO_2 in the ammonia*

solution and room air) at ordinary barometric pressure (750 to 770 mm.) and room temperature between 18–25° is identical within the error of the method (1 or 2 volumes per cent) with the figure obtained after making all corrections.

By subtracting the CO_2 in simple solution in the original blood (4) (3 volumes per cent at normal arterial and pulmonary CO_2 tension and temperature) a figure for the combined CO_2, or alkaline reserve, is obtained.

TABLE II.

Analysis of Sodium Carbonate Solution.

A solution of sodium carbonate from the dried recrystallized salt was prepared by weight. This was diluted as desired and 1 cc. of suitable concentration delivered into the diffusion tube and analyzed as for blood.

Temperature 22°, barometer 757 mm.

CO_2 as read from burette.	CO_2 after correction.	Theoretical CO_2.	Difference.
cc.	*vol. per cent*	*vol. per cent*	*vol. per cent*
1.08	109.0	111.0	−2
1.09	110.0	111.0	−1
0.90	90.0	89.0	+1
0.89	89.0	89.0	0
0.66	66.0	67.0	−1
0.66	66.0	67.0	−1
0.44	44.0	45.0	−1
0.45	45.0	45.0	0
0.21	20.0	22.0	−2
0.23	21.0	22.0	−1
0.12	12.0	11.0	+1
0.10	10.0	11.0	−1
0.01	1.0	0.0	+1
0.00	0.0	0.0	0

As a check on the accuracy of this method we have analyzed sodium carbonate and find that an accuracy within 2 volumes per cent of the theoretical is attainable. The results of a series of such analyses are given in Table II.

In many experiments we have employed the Van Slyke method as a check on that of Henderson and *vice versa*. The comparative results obtained are illustrated by the following figures, in which the first in each case is that by the former, the second that by the latter method.

Plasma: 53, 52; 57, 57; 61, 62; 54, 54; 59, 59; 71, 70; 62, 62; 37, 36; 35, 35; 30, 29; 22, 22; 17, 18; 7, 9; 6, 8; 19, 19; 19, 18; 14, 16; 59, 58; 55, 55; 52, 52; 44, 44; 44, 43; 43, 43; 32, 30; 27, 28; 17, 18; 20, 20; 22, 21; 19, 20; 23, 23; 18, 18; 23, 23; 21, 23; 62, 62; 59, 60; 49, 49; 33, 32; 47, 47; 51, 50; 39, 38.

Whole blood: 44, 46; 43, 42; 49, 47; 37, 37; 49, 49; 32, 32; 27, 29; 27, 30; 31, 31; 42, 44.

The CO_2 Content of Whole Blood.

The CO_2 content is determined by direct analysis of blood drawn from vein or artery without loss of CO_2. If the blood is collected rapidly under mineral oil and at once pipetted into the diffusion tube, no anticoagulant is necessary. For routine work, however, or where it is desirable to determine the CO_2 capacity, coagulation is prevented by powdered ammonium or sodium oxalate. This must be of absolute purity, and an amount should be used which will bring the total concentration to approximately 1 per cent. The technique for drawing and oxalating blood described by Van Slyke and Cullen (3) gives excellent results.

The CO_2 Capacity of Whole Blood.

The CO_2-combining power or capacity is estimated by determining the CO_2 content after the blood has been equilibrated with a definite tension of CO_2 under standard conditions of temperature and pressure. The tension of CO_2 now generally used for equilibration is that of the normal alveolar air; that is, 40 mm. partial pressure. We find that the employment of the last portion of the operator's expired air is inexact and involves large variations in CO_2 content when applied to whole blood. The air employed is best prepared by a modification of the Higgins-Plesch technique. A deep breath is expired into a bag and reinspired twice. The CO_2 tension of the air so obtained should always be established by analysis. The burette used for the blood determinations lends itself readily to this purpose. To get the tension in mm. the percentage of CO_2 found by analysis is multiplied by the barometer minus the vapor tension of water at the temperature at which the equilibration of the blood with this air is performed.

For the equilibration of whole blood the most satisfactory technique is the following. 5 cc. of blood free from mineral oil are pipetted into a 250 cc. Erlenmeyer flask tipped at an angle. The gas from the bag is passed slowly for 1 minute well beneath the surface of the fluid by means of a glass tube. With the gas still flowing and the flask full of foam, the tube is withdrawn and the stopper at once inserted. The flask is shaken continuously for at least 5 minutes with a rotary motion to spread the blood in a film over the surface and expedite the equilibrium which is complete at the end of 5 minutes.

In performing the saturation at a temperature higher than that of the surrounding air, the flask is immersed in a water bath. The increased pressure is relieved by loosening for a moment the edge of the stopper.

As regards the influence of temperature on the CO_2-combining power, or as we term it "the alkali called into use," in blood we have found that the effect varies in exact proportion to the amount of CO_2 dissolved at different temperatures. In other words, in a given blood put through a variety of temperatures without loss or gain of total CO_2, the tension of CO_2 varies, but the ratio of dissolved to combined CO_2 does not.

CONCLUSIONS.

1. In the Henderson-Morriss method of determining the CO_2 of plasma or whole blood it is found that the various corrections almost exactly neutralize each other. At ordinary temperatures and barometric pressures the reading on the gas burette for the CO_2 absorbed during the gas analysis (corrected only for the carbonate of the ammonia solution) may be taken as indicating directly, within the error of the method, the amount of CO_2 contained in the blood or plasma. Each 0.01 cc. corresponds to 1 volume per cent of CO_2.

2. This method is particularly valuable for the determination of the CO_2 content and combining power of whole blood. With it the analysis of whole blood is as easy as that of plasma.

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EXCHANGE OF CHLORIDE IONS AND OF CARBON DIOXIDE BETWEEN BLOOD CORPUSCLES AND BLOOD PLASMA.

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(Received for publication, February 10, 1920.)

Hamburger (1891) found that chlorides pass from the blood plasma into the red blood corpuscles when pure carbon dioxide is led through a sample of blood. In Hamburger's experiment, defibrinated blood was shaken with air, and the NaCl in the serum determined. Pure CO_2 was led through a sample of the same defibrinated blood and the amount of the NaCl in the serum fell from 0.58 to 0.53 per cent, 0.05 per cent having passed into the red corpuscles. The change proved to be reversible, the percentage of NaCl in serum increasing again when the CO_2-treated blood was shaken with air.

The experiment of Hamburger has been repeated several times with variations (Gürber, Koeppe, Petry, von Limbeck, and others). In his first paper Hamburger reported that in addition to chlorides other anions of the serum could pass into the corpuscles under the influence of CO_2, and this has recently been confirmed by new experiments of de Boer and of Hamburger himself (1918).

The experiments on exchange of anions between plasma and corpuscles have been criticized by Overton, who pointed out that in all the experiments the blood corpuscles had been treated with pure CO_2, and this procedure possibly had changed their permeability. Overton thought that uninjured blood corpuscles were impermeable for anions.

The next step is to determine what happens with the plasma chlorides if blood is saturated with different mixtures of carbon dioxide and air, especially whether or not a change in the tension

of CO_2 has any effect upon the distribution of the blood chlorides by low CO_2 tensions.

My first experiments deal with this point. In 1917 Van Slyke and Cullen in a paper dealing chiefly with other problems published experiments on the influence of different tensions of CO_2 on the distribution of the blood chlorides between plasma and corpuscles. My results are identical with theirs on some points. Still I shall briefly report my experiments on this point as an introduction to my further experiments and because I have used a different method.

Methods.

All experiments were carried out on fresh ox blood, the clotting being prevented by 0.1 per cent potassium oxalate. The volume of red blood corpuscles was estimated in an improvised hematocrit. The amount of hemoglobin was estimated by a standardized Haldane hemoglobinometer, the results being given in volumes per cent of oxygen capacity.

The saturation of blood samples with mixtures of air and carbon dioxide took place in saturaters of glass according to the method of Barcroft. Each saturater had a volume of 275 cc.; 3 cc. of blood were used for each experiment. One end of the saturater was closed by a stopper with a capillary glass tube through which samples of the air mixture in the saturater could be taken when the saturation of the blood was finished. The other end of the saturater was narrowed into a glass tube of the same diameter as a small centrifuge test-tube, being connected air-tight with this by means of a piece of rubber tube. After the saturation the blood was run into the centrifuge test-tube which was almost filled. After the piece of rubber tube had been pinched the centrifuge tube with the blood and the closed rubber tube as a stopper could be placed in a centrifuge without coming into contact with air other than that of the saturater.

The air sample from the saturater was analyzed in the Haldane apparatus.

The saturation took place by rotating the saturater in a water bath at 14–17°C. As no provisions were made to heat the centrifuge it was necessary to saturate at room temperature.

After centrifugation, samples of the blood plasma were taken for the determination of chlorides and eventually of carbon dioxide.[1]

The estimation of chlorides in the plasma was carried out after the micro method of Bang, not more than 0.1 cc. of blood being required for an estimation. I examined the accuracy of Bang's method in a series of experiments and found that the average error on a single estimation is 1.4 per cent of the total amount of chlorides. It is indifferent whether the amount of blood is measured in a calibrated pipette or weighed on a torsion balance, as Bang himself prefers. If the blood (or plasma) is determined by weight, a correction for loss by evaporation has to be introduced. In my experiments the blood was measured in a pipette. In every experiment I made three estimations of the amount of chlorides in the plasma, which reduces the average error to 0.6 per cent. Therefore variations of more than 2 per cent are real and cannot be ascribed to analytical errors. The second decimal is correct in estimations in blood or plasma (about 0.5 per cent of chlorides being found); the third is quite unreliable, but is given in the experimental accounts. The amounts of chlorides are conventionally given in percentage of NaCl, although this has certain disadvantages.

EXPERIMENTAL.

Exchange of Chloride Ions.

In Experiments 1, 2, and 3 blood was saturated with air containing different amounts of carbon dioxide, the chlorides being estimated in the plasma after the saturation.

Experiment 1.—Temperature 12°C.; Blood: oxygen capacity 18.3 volumes per cent, 0.418 per cent of NaCl.

CO_2 tension, *mm*	5.7	53.0	73.0	79.0
NaCl in plasma, *per cent*	0.528	0.510	0.512	0.498

[1] The estimations of CO_2 in blood and plasma were carried out by Mr. K. L. Gad-Andersen by the method of Haldane as modified by Dr. G. Lilliestrand in the laboratory of Professor A. Krogh. A paper by Dr. Lilliestrand describing the method will be published shortly.

Experiment 2.—Temperature 14°C.; Blood: oxygen capacity 15.9 volumes per cent, 0.436 per cent of NaCl.

CO_2 tension, *mm*.....	1.1	12.2	43.1	219.0	709.0
NaCl in plasma, *per cent*.....	0.591	0.551	0.526	0.509	0.479

Experiment 3.—Temperature 15.5°C.; Blood: oxygen capacity 15.2 volumes per cent, 0.460 per cent of NaCl.

CO_2 tension, *mm*.....	0.07	4.8	20.4	52.6	78.4
NaCl in plasma, *per cent*.....	0.600	0.577	0.552	0.543	0.535

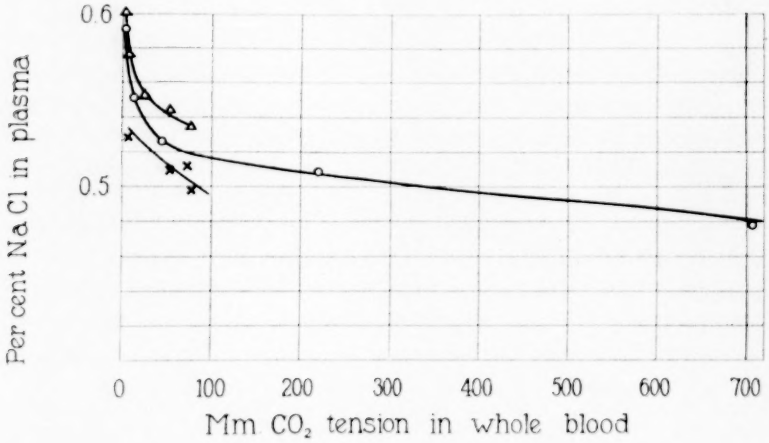

FIG. 1. Curves showing effects of varying amounts of CO_2 on the chlorides of the plasma. X = Experiment 1; O = Experiment 2; Δ = Experiment 3.

In Fig. 1 the results are drawn graphically (carbon dioxide tension as abscissæ and percentage of NaCl in plasma as ordinates).

The steep rise of the curve at the low CO_2 tensions refutes Overton's criticism of Hamburger's experiment. When a change in the CO_2 tension from 0.07 to 4.8 mm. makes a measurable quantity of chloride pass from the plasma into the corpuscles, it is impossible that the chloride transfer is due to an injury to the corpuscles by the carbon dioxide. At the high CO_2 tensions the curve is less steep but not parallel to the abscissa axis. The shape of the curve has much conformity with the inverse shape

of the curve representing the CO_2-combining power of the blood at various CO_2 tensions. Even at the high CO_2 tensions the plasma contains more chloride than the blood; that is, the chloride content of the corpuscles is never so great as that of the plasma. The distribution of chloride in the blood is never uniform.

The results make it evident that estimation of chlorides in the plasma or serum from blood which has been kept in open receivers must give too high results, because chlorides have passed into the plasma (or serum) on account of the decreasing CO_2 tension.

Experiments 4 and 5 deal with the question of whether the cause of the wandering of the blood chlorides lies in the plasma or in the corpuscles. The problem was attacked by comparing the influence of changes in CO_2 tension upon a sample of blood and upon a sample of the same blood diluted with its own plasma. The plasma for dilution was produced by centrifuging the blood at about 40 mm. CO_2 tension.

Experiment 4.—Temperature 18.5°C.; Sample A: 44 volumes per cent blood corpuscles, oxygen capacity 19.9 volumes per cent, 0.474 per cent of NaCl.

By centrifuging at 40 mm. CO_2 tension plasma is prepared from Sample A.

Sample B is prepared by adding 1 volume of Sample A to 1 volume of its plasma. Sample B: 22 volumes per cent blood corpuscles, oxygen capacity 9.9 volumes per cent, 0.522 per cent of NaCl.

CO_2 tension, *mm*	41.9	47.3	602	681
NaCl in plasma from Sample A after saturating, *per cent*	0.564		0.509	
NaCl in plasma from Sample B after saturating,* *per cent*		0.565		0.545
NaCl in blood corpuscles from Sample A after saturating,* *per cent*	0.36		0.43	
NaCl in blood corpuscles from Sample B after saturating,* *per cent*		0.36		0.43

* The percentage of NaCl in the red blood corpuscles is calculated from the percentage of NaCl of the blood and the plasma by means of the known volume of the red corpuscles (estimated by the hematocrit).

Experiment 5.—Temperature 14°C.; Sample A: 33 volumes per cent blood corpuscles, oxygen capacity 15.0 volumes per cent, 0.442 per cent of NaCl.

Sample B prepared by adding 1 volume of Sample A to 2 volumes of its own plasma. Sample B: 11 volumes per cent blood corpuscles, oxygen capacity 5.0 volumes per cent, 0.483 per cent of NaCl.

CO_2 tension, *mm*	1.2	1.6	34.3	36.7	505	694
NaCl in plasma from Sample A, *per cent*	0.536		0.511		0.460	
NaCl in plasma from Sample B, *per cent*		0.519		0.500		0.491
NaCl in corpuscles from Sample A, *per cent*	0.25		0.31		0.41	
NaCl in corpuscles from Sample B, *per cent*		0.27		0.32		0.42

In Experiment 4 the percentage of NaCl at about 40 mm. CO_2 tension was the same in plasma from the original blood (Sample A) and in plasma from the diluted blood (Sample B). In the corpuscles the percentage of NaCl also was the same in Samples A and B at about 40 mm. CO_2 tension. When the two samples of blood were saturated with a much higher CO_2 tension (about 600 to 680 mm.) the percentage of NaCl fell in the plasma of both, but much more in the plasma from the original blood, Sample A (from 0.564 to 0.509 per cent), than in the plasma of the diluted blood, Sample B (from 0.565 to 0.545 per cent). In the red corpuscles, on the contrary, the percentage of NaCl rose with an equal amount in corpuscles from original and from diluted blood (in either from 0.36 to 0.43 per cent).

Experiment 5 shows the same, and furthermore that by lowering the CO_2 tension (from 35 to 1 mm.) the percentage of NaCl rose more in plasma from the original (from 0.511 to 0.536 per cent) than in plasma from the diluted blood (from 0.500 to 0.519 per cent), but coincident with this the percentage of NaCl of the corpuscles fell in each sample by an equal amount.

These results make it evident that the shifting of the blood chlorides is caused by some properties of the red blood corpuscles. A given change in the CO_2 tension always produces a given change in the percentage of NaCl of the blood corpuscles, and the percentage of NaCl in the plasma is secondarily regulated by this.

An objection could be raised against the calculation of the percentage of NaCl of the corpuscles, Hamburger (1902) having proved that a change in CO_2 tension affects the volume of the corpuscles. But even a considerable error in the estimation of the volume of the blood corpuscles would not interfere with the results to any noticeable degree.

The cause of the exchange of chloride between blood plasma and blood corpuscles must be looked for in the red corpuscles. In the next section we will consider which constituent of the red corpuscles is involved in this reaction.

Coincident Exchange of Carbon Dioxide and Chloride Ions.

In a recent paper Hasselbalch proved that the hydrogen ion concentration of the blood at a given CO_2 tension is identical with that of a solution of sodium bicarbonate containing the same amount of combined carbon dioxide as the blood and under the same CO_2 tension. But with a change in the CO_2 tension the blood behaves differently from a solution of sodium bicarbonate. The sodium bicarbonate solution contains the same amount of combined carbon dioxide at all CO_2 tensions, but at extremely low tensions some monocarbonate is formed. In blood, however, the amount of combined carbon dioxide increases with increasing CO_2 tension as if the amount of bicarbonate in the blood were increasing too. Hasselbalch explains this by the physicochemical qualities of hemoglobin acting as an amphoteric electrolyte, which has acid qualities so that at low CO_2 tensions it can displace carbon dioxide from the bicarbonate. At high CO_2 tensions hemoglobin assumes alkaline qualities and combines with carbon dioxide in amounts that increase with the CO_2 tension.

Hasselbalch's experiments were performed with solutions of hemoglobin and sodium bicarbonate. The question is how the hemoglobin in the blood can influence the bicarbonate of the plasma as in a solution of hemoglobin, while in the blood the hemoglobin is confined to the corpuscles. This question is explained by the fact that the chloride ions and other anions represent a link between the constituents of the corpuscles and those of the plasma.

When an increase of the CO_2 tension makes chloride pass from the plasma to the corpuscles, the plasma coincidently with this turns more alkaline, and consequently can combine with more carbon dioxide. Gürber has explained this fact in the following way: The carbon dioxide sets free a certain amount of HCl from the chlorides of the plasma; this HCl passes into the corpuscles and leaves the basic components of the chlorides in the plasma to combine with more carbon dioxide. The explanation of Gürber translated into the language of modern chemistry might be worded as follows: Carbon dioxide displaces anions, especially chloride ions, from plasma into corpuscles. Coincidentally with this an equivalent amount of other anions, *viz.* hydroxyl ions, passes from the corpuscles into the plasma. This exchange turns a part of the NaCl of the plasma into NaOH, which combines with some of the free carbon dioxide to form bicarbonate. Koeppe and Hamburger (1902) express the same thing in another way which I do not prefer.

Of course other explanations are possible, especially since Hamburger and Bubanovic have shown that it is likely that the blood corpuscles are permeable not only to anions but also to cations. The matter can be settled by experiments dealing with the minute quantitative relation between chloride and bicarbonate in plasma and corpuscles.

Experiment 6.—Blood was saturated with air at a very low CO_2 tension (0.08 mm.) and centrifuged. Consequently the plasma was very rich in chloride (0.580 per cent of NaCl). Then a sample of the plasma and a sample of the original blood were saturated at a high CO_2 tension (162 mm.). Of course this did not affect the percentage of NaCl of the plasma, but in the blood the plasma became poorer in chloride (0.476 per cent of NaCl), 0.104 per cent of NaCl having passed into the corpuscles.

	NaCl	CO_2
	per cent	*vol. per cent*
Blood at 0.08 mm. CO_2 tension, in the plasma.....	0.580	
Plasma " 163 " CO_2 "	0.580	55.6
Blood " 162 " CO_2 " in the plasma..	0.476	103.0
Difference.....	−0.104	+47.4

At 163 mm. CO_2 tension the plasma contained 55.6 volumes per cent of CO_2. But the blood saturated at the same CO_2 tension (162 mm.) contained in its plasma (not in the entire blood) 103.0 volumes per cent of CO_2; *i.e.*, 47.4 volumes per cent of CO_2 more than the original plasma separated from the corpuscles at the low CO_2 tension contained at the same CO_2 tension. In going from the low to the high CO_2 tension, 0.104 per cent of NaCl disappeared from the plasma. But coincidently with this the plasma was able to bind 47.4 volumes per cent of CO_2 more. 0.104 per cent of NaCl is equivalent to 39.4 volumes per cent of CO_2; that is, the NaOH formed by the disappearance of chloride ions from the plasma almost completely accounts for the increased amount of carbon dioxide with which the plasma was able to combine.

In Experiment 6 the chloride ions displaced by carbon dioxide from the plasma almost completely account for the increased carbon dioxide-combining power gained by the plasma, but not quite completely, 8 volumes per cent of CO_2 failing to be accounted for. This small amount is probably accounted for by anions other than chlorides passing from the plasma into the corpuscles (de Boer, Hamburger (1918)).

In some experiments on the same problem, Van Slyke and Cullen[2] found that the Cl change did not so closely approximate the CO_2 change. In their experiments other methods were used, and the CO_2 tension in one determination was computed and not gained by analysis. But neither of these facts can account for the considerable difference in the results, which at present I am unable to explain.

If my result holds good, it explains in what way the hemoglobin, although confined to the corpuscle, can influence the CO_2-combining power of the plasma. When the plasma at higher CO_2 tensions binds more CO_2 it is due to the anions passing into the corpuscles to be bound by the hemoglobin, leaving the cations in the plasma to combine with an amount of carbon dioxide equivalent to the amount of disappeared anions.

In Experiment 7 the distribution of chlorides and carbon dioxide at different CO_2 tensions between plasma and corpuscles has been estimated, the NaCl and the bound CO_2 being estimated in the plasma as well as in the blood. The amounts in the corpuscles were calculated from these estimations and from the known volume of the corpuscles. Furthermore the hydrogen ion

[2] Van Slyke and Cullen, pp. 343, 344.

concentration has been calculated for blood, plasma, and corpuscles by the method of Hasselbalch from the amounts of free and of bound carbon dioxide.

Experiment 7.—Temperature 17°C.; 38 volumes per cent blood corpuscles.

CO_2 tension, *mm*		0.08	6.1	39.1
NaCl in	blood, *per cent*	0.456	0.456	0.456
	plasma, *per cent*	0.578	0.533	0.511
	corpuscles, *per cent*	0.260	0.334	0.369
CO_2 bound in	blood, *vol. per cent*	20.8	37.9	65.5
	plasma, *vol. per cent*	23.4	42.6	67.6
	corpuscles, *vol. per cent*	16.6	27.6	62.0
pH in	blood	9.61	7.92	7.31
	plasma	9.64	7.94	7.35
	corpuscles	9.57	7.85	7.30

Experiment 7 shows, as the preceding ones, that with increasing CO_2 tension the amount of NaCl in the plasma decreases and the amount of CO_2 increases. But Experiment 7 shows a new fact too; namely, that not only does the percentage of NaCl in the blood corpuscles increase with increasing CO_2 tension, as is already known, but the amount of carbon dioxide in the corpuscles also increases with increasing CO_2 tension. Although anions at the higher CO_2 tensions pass into the corpuscles the latter do not, as might be expected, become more acid, but on the contrary more alkaline, being able to combine with more carbon dioxide. In the plasma more bicarbonate is generated by the disappearance of anions. In spite of the fact that the corpuscles received the anions, the amount of bicarbonate in the corpuscles apparently increased too. As a result of these changes the hydrogen ion concentration at the CO_2 tensions of the experiments is almost constant in the plasma and the corpuscles, the corpuscles being a little more acid. At very high CO_2 tensions I have not succeeded in getting reliable results.

The explanation of Gürber evidently does not suffice to settle the problem. But when this explanation is combined with Hasselbalch's interpretation of the hemoglobin as an amphoteric

electrolyte, the solution of the problem is simple. The more the CO_2 tension (and the hydrogen ion concentration) increases, the more the hemoglobin turns alkaline and the more its power to combine with CO_2 increases. But carbon dioxide ions are anions, as are the chloride ions, and the same property of the hemoglobin which explains its behavior toward carbon dioxide explains also the exchange of chloride ions. Chloride ions and carbon dioxide passing into the corpuscles at increasing hydrogen ion concentration, *viz.* CO_2 tension, are parallel phenomena and due to the same property of the hemoglobin. The difference is due only to the behavior of the plasma, which in a saturater is able to combine with new carbon dioxide from the air of the saturater, replacing the amount which has passed into corpuscles; the plasma is not able to replace the amount of chloride ions which have disappeared, because chloride ions do not exist in the air. This is the explanation, too, why the shape of the curve representing the exchange of chloride ions at different CO_2 tensions as mentioned has conformity with the inverse shape of the curve representing the CO_2-combining power of the blood at various CO_2 tensions.

In a recently published paper[3] Hasselbalch and Warburg fully agree with this interpretation of the facts.

I have made some experiments to see if the chlorides had any influence upon the total amount of carbon dioxide with which the hemoglobin can combine. Experiment 8 is typical.

Experiment 8.—Red blood corpuscles were deprived of their content of chlorides by washing three times with a solution holding 7.3 per cent of cane sugar and 0.185 per cent of sodium bicarbonate. Previous experiments had shown it impossible to deprive corpuscles of all chlorides by washing with isotonic cane sugar solution without addition of a slight amount of bicarbonate to make the solution faintly alkaline. Then the bicarbonate adhering externally to the corpuscles was removed by washing three times with 8.5 per cent cane sugar solution. An analysis was then made and showed no trace of chlorine in the corpuscles. To 32 cc. of the suspension of corpuscles were added 10 cc. of 1.3 per cent sodium bicarbonate solution. This was called Mixture A.

To 20 cc. of Mixture A were added 10 cc. of 8.5 per cent cane sugar solution. This was called Mixture B.

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[3] Read at the same meeting of the Biological Society of Copenhagen as the present paper.

To 20 cc. of Mixture A were added 10 cc. of 0.9 per cent NaCl solution. This was called Mixture C.

Mixtures B and C contained 53 volumes per cent of carbon dioxide combined as bicarbonate.

Samples of Mixtures A and B were then saturated at 17°C. with air containing 29 per cent of carbon dioxide, and other samples with atmospheric air containing 0.03 per cent of carbon dioxide. The amount of carbon dioxide in the various samples was estimated.

CO_2 tension, *mm*	0.2	218.0
CO_2 in Mixture B, *vol. per cent*	53.9	135.5
CO_2 " " C " " "	51.2	142.6

The results show that chloride-free red blood corpuscles combine with about the same total amount of CO_2 at various CO_2 tensions whether suspended in cane sugar solution or in NaCl solution.

Chlorides in the plasma interfere only with the distribution of carbon dioxide between plasma and corpuscles and not appreciably with the total amount of carbon dioxide, with which chloride-free corpuscles (and presumably the blood) are able to combine.

SUMMARY.

1. The influence of various CO_2 tensions upon the distribution of chlorides between blood plasma and red blood corpuscles was experimentally investigated and the results are represented in curves showing conformity with the curve representing the CO_2-combining power of the blood at various CO_2 tensions.

2. I have been able to confirm, with a different method, the results of Van Slyke and Cullen's experiments on the influence of the CO_2 tension on the partition of the chlorides between plasma and red blood corpuscles.

3. Experimental evidence is presented to show that the cause of the exchange of chloride ions and other anions between plasma and corpuscles is in the red corpuscles and not in the plasma.

4. The amount of chloride ions displaced from the plasma into the corpuscles by increasing CO_2 tension from 0.08 to 162 mm. almost completely accounts for the increased CO_2-combining power coincidently gained by the plasma. My experiments on this point (Experiment 6) do not agree quantitatively with those of Van Slyke and Cullen.

5. Increasing CO_2 tension increases both the CO_2-combining power of the plasma and that of the red corpuscles. The hydrogen ion concentration remains almost the same in plasma and corpuscles at different CO_2 tensions; it is only slightly higher in the corpuscles than in the plasma.

6. If the explanation of Hasselbalch of the properties of the hemoglobin is accepted, it is pointed out that this interpretation explains not only the increasing CO_2-combining power of hemoglobin at increasing CO_2 tension, but also the passing into the red corpuscles of chloride ions (and other anions) at increasing CO_2 tension, the two phenomena being parallel and due to the same property of the hemoglobin. This interpretation accounts for all observed facts.

7. The chlorides of the blood influence only the distribution of carbon dioxide between plasma and red corpuscles, and not appreciably the total amount of carbon dioxide with which the blood is able to combine at various CO_2 tensions.

I wish to thank Professor A. Krogh for much advice and assistance in the experiments.

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A QUANTITATIVE METHOD FOR DETERMINATION OF VITAMINE.

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(Received for publication, March 5, 1920.)

INTRODUCTION.

In a previous paper[1] it was shown that yeast needs for its nutrition a substance of unknown nature, and evidence was presented that this substance is the beri-beri-preventing vitamine. Shortly after, Bachmann[2] published a paper in which the same conclusion was drawn using a different method of study. It was stated that not only does the growth of single yeast cells indicate presence of vitamine but the rate of growth is dependent on the quantity of vitamine present, and hence it was hoped that the method would be of quantitative value. As a matter of fact, the number of cells produced from one in 18 hours under given conditions is directly proportional to the amount of vitamine added. This test, while of value quantitatively, having been used successfully by a number of people, is not ideal in that it requires some practice to apply it; it introduces a personal factor to quite an extent and is a strain on the eyes if applied regularly. A method is now presented which is based upon a gravimetric determination and measures the growth of a very large number of cells rather than a few which have been isolated, and hence the variations in results are not so large.

The gravimetric method is more accurate than the single cell method; it is more easily applied and the personal factor is largely eliminated. An outline of this method is given first, followed by the justification of the various steps and a general discussion of its application.

[1] Williams, R. J., *J. Biol. Chem.*, 1919, xxxviii, 465.

[2] Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.

Method.

The following solution is used as a stock solution:

20.0 gm. of cane sugar.	1.5 gm. of asparagine.
3.0 " " $(NH_4)_2SO_4$.	0.25 " " $CaCl_2$.
2.0 " " KH_2PO_4.	0.25 " " $MgSO_4$.

All dissolved in 1 liter of distilled water.

100 cc. of this solution are put into a 500 cc. Erlenmeyer flask and to it is added a known amount of the solution to be tested. The volume is then made up to 110 cc., the flask is plugged with cotton, sterilized or pasteurized to kill all vegetative organisms, cooled, and its temperature brought to 30°C. in an incubator.

A yeast suspension is made by weighing out 0.300 gm. of fresh Fleischmann's yeast (small cake in tin-foil) taken from the center of the cake; this is made into a paste with a very small amount of water and suspended finally in 1 liter of sterile water. 1 cc. of this suspension well shaken and freshly made is introduced into the culture medium with a sterile pipette. By this means 0.3 mg. of yeast is used for seeding.

The flasks are then put into the incubator and the yeast is allowed to grow undisturbed 18 hours at 30°C., when the growth is stopped by the addition of a little formaldehyde solution. If the seed yeast has not been kept in a refrigerator continuously since manufacture, a very rapidly growing wild yeast may appear to a slight extent, floating on the surface of the solution. Practically all this should be removed by lifting it from the surface with a small piece of fine copper gauze attached at right angles on the end of a glass rod or tube. This removes practically none of the other yeast, most of which settles to the bottom.

After removing the wild yeast, if any is present, the yeast is filtered off on a weighed Gooch crucible, prepared with paper underneath the asbestos, washed thoroughly with water, finally with a little alcohol, dried for 2 hours at 103°C., and weighed after an hour's cooling. The Gooch crucibles cannot be used repeatedly without treatment as the dried yeast loses weight on washing with water.

If no vitamine is added to the solution the yeast produced will be about 2.5 mg. The amount of growth above that of the blank

is, within limits, directly proportional to the amount of vitamine put into the solution. The curve in Fig. 1 indicates the character of the results obtained in the first experiments carried out according to the method outlined.

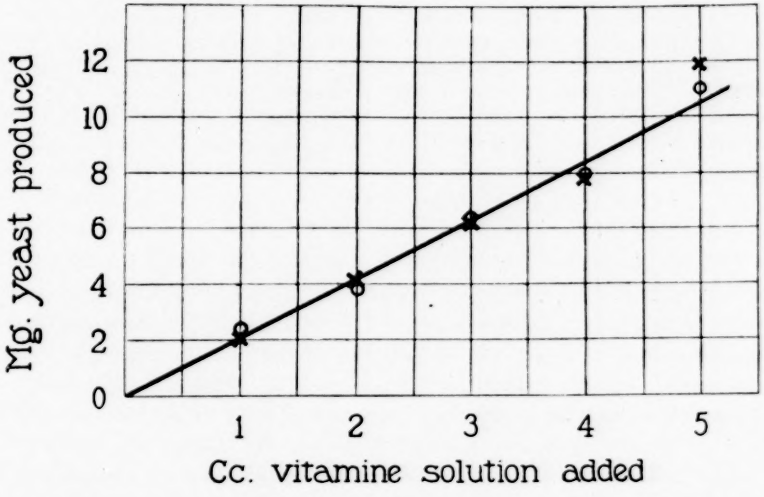

FIG. 1. The points marked with a cross and those marked with a circle were obtained on different days with different samples of yeast. The "Mg. yeast produced" applies to the amount after subtraction of the blank. The "Vitamine solution" contained 1.5 mg. of crude material per cc. For some distance above the upper limits of the curve there is still a proportionality, provided the material tested is free from inhibiting substances.

Expression of Results.

The results may be expressed numerically and the number represents the relative purity of the preparation tested or the value of the material tested as a source of this vitamine. The "vitamine number" of a material may be defined as the number of milligrams of yeast produced by the addition of its extract minus that produced in a control solution, under given conditions and within certain limits, computed to 1 gm. of the original material tested.

Thus if 0.100 gm. of a preparation produces 8.0 mg. of yeast more than a control, its vitamine number computed to 1 gm. is 80. If 0.05 gm. of the preparation is used as a duplicate, approximately 4.0 mg. more yeast are produced than in the control. Computing this to 1 gm., the vitamine number of the preparation is 80, the same as before. It is obvious that this form of expression of results makes possible the comparison of various preparations made at different times and in different localities. By simple water extraction of yeast (such as that used for seeding) and testing the extract obtained we get a vitamine number computed to 1 gm. of dry yeast, which is in the neighborhood of 500. Protein-free milk gives a value of about 12 computed to 1 gm. of the original milk, or 92 computed to the original milk solids. These are merely examples showing the order of magnitude of the numbers.

DISCUSSION.

By watching the growth of single cells under given conditions, which need not be explained in detail, results were obtained which were quantitative in character. The value of a preparation can be expressed in terms of the number of cells produced from one cell under certain conditions, calculated to 1 gm. of substance. The term "vitamine number" as used above is approximately one-twentieth of this value obtained by the single cell method.

By this method of study a number of facts were discovered which have an important bearing on the method under discussion. It has long been supposed that the addition of protein-split products to a medium containing ammonium salt as the sole source of nitrogen improves the medium for yeast. I have found it very difficult to prepare a protein digest free from vitamine which can be made uniformly, and which is a favorable nutrient for yeast. Enzymes cannot be safely used for hydrolysis for this purpose as the enzyme preparations may contain a considerable amount of vitamine. On the other hand, it was found by watching single cells that when ammonium sulfate is supplemented with organic nitrogen, free from vitamine, faster growth may result in the presence of a given amount of vitamine than without the organic nitrogen. It was found that accelerated growth is produced by the addition of a small amount of asparagine to the medium containing ammonium sulfate. The asparagine furnishes amide and amino nitrogen in addition to the ammonia nitrogen already present. A solution which contains ammonium sulfate *and* asparagine is not improved by the addition of a mix-

ture of amino-acids. In an experiment with the single cell method, a mixture of purified amino-acids, alanine, leucine, tyrosine, aspartic acid, glutaminic acid, cystine, histidine, and tryptophane, was added to a medium containing asparagine and a small amount of vitamine to see if the amino-acids improved the medium. The blank with no amino-acids present produced an average of 120 cells from one cell in 18 hours. With 5.0 mg. of the amino-acid mixture the average growth was 108 cells and with 25.0 mg. the average was 96 cells. From these results the conclusion was drawn that nothing except vitamine in the small amounts used will materially improve the medium which already contains asparagine in addition to ammonium sulfate.

It was found by studying the growth of single cells that if an 18 hour incubation period was allowed the growth in this time is directly proportional to the amount of vitamine put into the medium. The 18 hour period was successfully carried over to the gravimetric method here outlined.

When the single cell method is used, commercial yeast can be used to very great advantage, being remarkably uniform in strength and easily suspended for the isolation of single cells. For the gravimetric method also commercial pressed yeast with uniform vitality, moisture content, etc. is very valuable as it makes possible a uniform seeding of the media both in quantity and quality of the yeast. Obviously no bakers' yeast is absolutely free from foreign organisms, but the foreign organisms are very few and offer no serious handicap, as the results indicate. The wild yeast referred to which occasionally appears on the surface of the solution would not have to be considered if it was not for the fact that it multiplies with enormous rapidity even in a weak medium. It does not appear even when quite old yeast is used, if the yeast has been kept at low temperature.

The method as here outlined applies to solutions; that is, the vitamine to be tested must be in solution. No method of extraction is outlined. The fact that nothing except vitamine (especially in small amounts) is able to produce more rapid growth shows that the vitamine does not need to be prepared especially free from other materials, as impurities in all probability will have no accelerating effect. The total amount of material added ordinarily will be much less than 0.1 gm. In using this method for vitamine determinations precautions must be taken that the

vitamine is extracted quantitatively from the material in question. Investigators have found that this is difficult to do, especially from some materials. In the handling of the extracts care must be taken that toxic substances are not introduced or formed which will counteract the effect of the vitamine which may be present. This is true also of animal feeding experiments where poisonous substances must be avoided. Treatment of material with acid especially at high temperature or overheating in the dry condition frequently produces something toxic to yeast, while treatment with alkali may destroy the vitamine. The vitamine in some extracts is destroyed to an appreciable extent if kept at the boiling temperature an hour or more. An autolysate of yeast is not a desirable material as a yeast nutrient as inhibiting substances appear to be present. If the amount of growth produced is not proportional to the material added, but falls off with the larger quantity, it is good evidence that something toxic is present, which becomes more potent in the higher concentration and counteracts the effect of the increase in vitamine. This can be confirmed by microscopic examination of the yeast produced, which reveals small granular irregular cells or other abnormalities. It is mainly due to the possible presence of toxic substances that two dilutions should be used for a determination. If toxic substances are present the results from the lower concentrations are more reliable.

It is believed that this method will be of value in the solution of various problems connected with this vitamine. The ultimate source of this water-soluble vitamine, whether it is the green plant or the bacteria of the soil or other organisms, is not settled. Recently, however, evidence has been obtained that when yeast is provided with a certain amount of vitamine it is unquestionably able to build up more. In other words it has some synthetic powers which have also been attributed to animals. The function of this vitamine in animal and plant life, where it seems to occur almost universally, is very obscure. It is a suggestive fact, however, that in some of its properties, solubility, heat stability, alkali destruction, dialyzability, etc., this vitamine bears a resemblance to the coenzyme of alcoholic fermentation.[3]

[3] For general discussion see Harden, A., Alcoholic fermentation, Monographs on biochemistry, London, New York, Bombay, and Calcutta, 1911, 59-69.

SUMMARY.

A quantitative method for determination of the antineuritic or water-soluble vitamine is presented, which is based upon a gravimetric determination.

A numerical expression of results is suggested whereby the vitamine content of any material can be rated in terms of milligrams of yeast produced under given conditions.

I wish to express thanks to Professor F. C. Koch and Dr. R. E. Lee for help given in carrying out this work.

MODIFICATION OF THE VAN SLYKE METHOD FOR DETERMINING ARGININE.*

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(Received for publication, April 14, 1920.)

Confirming the observation of Osborne, Leavenworth, and Brautlecht[1] that arginine liberates one-half of its nitrogen as ammonia when boiled with concentrated alkali, Van Slyke[2] has made use of a Folin bulb set on top of a tall Liebig condenser to absorb and confine to the system such ammonia as is liberated during the boiling. This method has been used extensively in this laboratory, but from time to time there has been occasion to note the difficulties entailed in its application. The most obvious disadvantage is the possibility of bumping which is likely to occur when such a concentrated solution is boiled for any great length of time. When it does occur, the standard acid in the bulb may be ejected from the bulb or be drawn back into the condenser.

To eliminate these objections and especially to facilitate the securing of accurate results, the apparatus shown in Fig. 1 has been devised and applied with much success. By its use, bumping is entirely eliminated and the ammonia as formed is drawn over into the standard acid by a *slow* current of air. The excess of acid can thus be titrated at once at the termination of the digestion. By the use of N/14 acid, values in cc. synonymous with mg. of nitrogen are obtained directly. In the aspiration it is necessary to use care not to mistake passage of the air through the alkali for ebullition and not to aspirate at too rapid a rate. In other respects the technique used by Van Slyke has been closely followed.

*Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Osborne, T. B., Leavenworth, C. S., and Brautlecht, C. A., *Am. J. Physiol.*, 1908–09, xxiii, 194.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15; 1915, xxiii, 411.

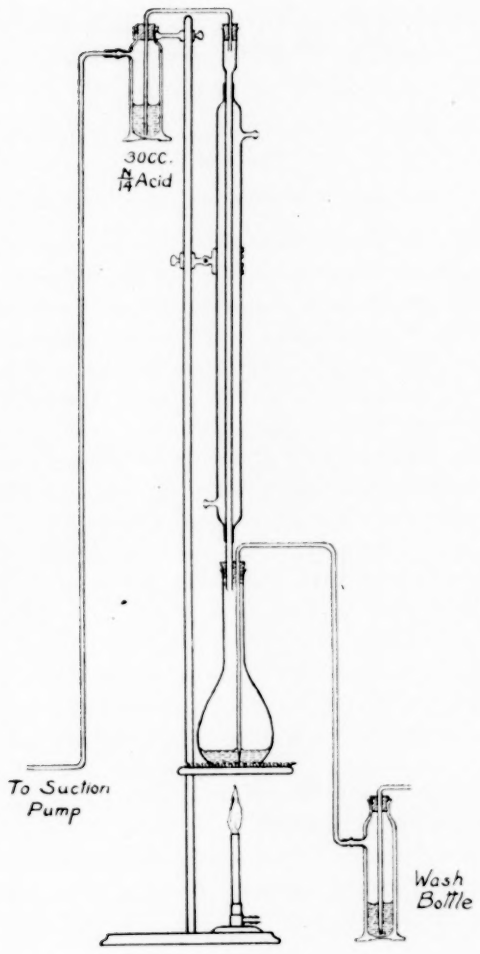

FIG. 1.

FURTHER DATA CONCERNING THE ALLEGED RELATION OF CATALASE TO ANIMAL OXIDATIONS.

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(Received for publication, April 15, 1920.)

Not long since one of us (1) presented evidence which indicated that the causal relation between the catalase concentration of the blood and the intensity of animal metabolism, alleged by Burge (2) to exist, was far from being conclusively demonstrated by his data. A simpler explanation supported by experimental results was presented.

Burge's conclusions were drawn from a certain parallelism existing between the factors noted. As far as the authors are aware he has made no measurements of the intensity of metabolic activity in the course of any of his experiments. It may be true that metabolic intensity and catalase content do vary in the same direction under the conditions he describes but in many and possibly all of his experiments in which these parallel variations in catalase content and metabolic intensity are in harmony with his idea an additional variable factor is introduced; *viz.*, the number of red cells. Inasmuch as the catalase of the blood is contained in the cells, particularly in the erythrocytes, any significance that Burge's experiments really possess is that under the conditions imposed by him the red cell content of the blood varies and with it the catalase as a necessary consequence. The variations in catalase content are, therefore, secondary in nature and bear no necessary relation to the rate of metabolism. This is very different from the unwarranted conclusion that catalase is the oxidizing enzyme of the body.

It is possible to vary the intensity of animal metabolism to a great extent without observing any noteworthy change in the red cell content of the blood. This being true it becomes possible

to test Burge's conclusions directly by determining whether, as a matter of fact, there is any change in the catalase content of the blood when the red cell count is constant and the rate of metabolism varied. The result of such a test is the reason for this communication.

Measurements were made of the carbon dioxide production and hemoglobin and catalase contents of rabbit and cat blood drawn to correspond as nearly as possible to successive periods of normal and high metabolism. The apparatus described by Haldane (3) was employed for the carbon dioxide determinations. The high metabolism of the one period was induced in the case of the rabbits by surrounding the animal chamber with ice and partially wetting the fur of the animals and in the case of the cat experiment by the use of a freezing mixture only. Before the beginning of the collection of carbon dioxide, air was drawn through the apparatus with the animal in place for from 11 to 15 minutes. The current varied between 4 and 6 liters per minute in the different experiments but the rate was practically constant in any one of them. Thus the accumulation of carbon dioxide in the chamber was prevented since in the fore periods and in the intermediate period of the last experiment there was ample opportunity for an equilibrium to be established between the production and removal of the gas. Therefore the carbon dioxide collected in the subsequent periods closely approximates the actual production in those periods. In the rabbit experiments the period of high metabolism followed that of normal metabolism while in the cat experiment the order was reversed. The animals varied in size, which accounts partly for the differences in the rates of metabolism noted. They filled the chamber almost completely so that there was very little voluntary activity. Catalase estimations were made by the method already described (1); hemoglobin determinations were made by Palmer's (4) procedure. The results of the experiments are shown in Table I.

These six experiments appear to demonstrate conclusively that there may be great variations in the rate of animal metabolism without any corresponding change in the catalase content of the blood. According to Burge's hypothesis variations in catalase content and carbon dioxide production should be parallel. The statement is warranted, therefore, that the paral-

lelism between metabolic intensity and the catalase content of blood which sometimes exists, but which is not found here, does not prove any intimate relation between the two. On the other hand, it appears that the data here presented suggest the absence of such an interrelation in so far as it is reasonable to expect varia-

TABLE I.

Animal.	Period.	Catalase.	Hemoglobin.	CO_2	Remarks.
		cc.	*per cent*	*gm.*	
Rabbit 1.	Room temperature.	131,130	84	1.34	45 min. periods. Blood drawn from ear immediately preceding Period A and at end of Period B.
	Low "	133,130	90	3.56	
" 2.	Room "	115,116	83	0.69	30 min. periods. Blood as above.
	Low "	125,129	83	2.16	
" 3.	Room "	124,122	71	1.18	30 min. periods. Blood as above. Temperature of chamber lowered further in Period B.
	Low " (Period A)	127,117	71	1.69	
	Low temperature. (Period B)	120,121	74	2.20	
" 4.	Room temperature.	142,136	74	1.70	45 min. periods. Blood as above.
	Low "	128,150	71	2.17	
" 5.	Room "	173,167	96	1.10	30 min. periods. Blood as above.
	Low "	166,170	95	2.20	
Cat 1.	Low "	212,213	76	4.06	45 min. periods. Blood drawn from femoral vein at end of each period.
	Room "	235,242	81	1.95	

tions in the oxidizing capacity of the tissues to be reflected in the concentration of oxidizing enzymes in the blood.[1]

[1] Since this paper was written Seymour has reported that no direct relation exists between the catalase content of turtle heart muscle and the rate of its beat (Seymour, R. J., *Am. J. Physiol.*, 1920, li, 525).

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THE FERMENTATION OF GLUCOSE, GALACTOSE, AND MANNOSE BY LACTOBACILLUS PENTOACETICUS, N. SP.*

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(Received for publication, April 22, 1920.)

The pentose-fermenting bacteria to which we have given the name *Lactobacillus pentoaceticus, n. sp.*[1] exhibit a wide range of activity, both with respect to carbohydrates fermented and products formed. The pentoses, xylose and arabinose, are rapidly and completely broken down to acetic acid, lactic acid, and only the merest trace of other products such as carbon dioxide and alcohol. The fermentation of fructose is not so simple and results in the production of large quantities of carbon dioxide and mannitol in addition to the products formed from xylose.[2] The fermentation of fructose to mannitol is completed in 4 or 5 days, and a secondary fermentation of the mannitol then ensues, the chief products of which appear to be much the same as those derived from fructose. However, the fermentation of mannitol is much slower than that of fructose.

The fermentation of the aldo-hexoses, glucose, galactose, and mannose, differs from the fermentation of fructose in that no mannitol and very little acetic acid are formed, but a new compound, ethyl alcohol, is produced in large quantities. In old cultures a part of the lactic acid formed may be broken down to

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

This work was in part supported by a grant from the special research fund of the University of Wisconsin.

[1] Fred, E. B., Peterson, W. H., and Davenport, A., *J. Biol. Chem.*, 1919, xxxix, 347.

[2] Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, 1920, xli, 431.

acetic acid and carbon dioxide as decomposition products. On the basis of the quantitative data obtained, it is proposed that the main line of the fermentation for the aldo-hexoses may be represented by the equation

$$C_6H_{12}O_6 = CH_3CHOH.COOH + C_2H_5OH + CO_2$$

The small amount of acetic acid found probably results from a secondary fermentation of the lactic acid. The application of quantitative data to this equation will be considered in the experimental part of this paper.

The products formed from the aldo-hexoses by the pentose fermenters, *Lactobacillus pentoaceticus*, indicate a relationship to other organisms such as the "mannite-bacteria" of Gayon and Dubourg, Laborde, Müller-Thurgau and Osterwalder, and *Bacillus coli communis* of Harden, and the lactic acid bacteria of Kayser.[3]

Complete data for all the products formed by different bacteria are meager. Most of the investigational work has been concerned with a measurement of one or two products rather than a balance of all the products. It is, therefore, often difficult to determine the underlying causes that modify the formation of a given product. Frequently the variation in amount of one product is intimately associated with variation in the amount of another product. This interrelation between the products is exhibited very strikingly in the recent work[4] with yeast where glycerol production is paired with the production of acetaldehyde or acetic acid. Likewise, compounds which are termed final products may be the result of secondary fermentations, so that it is impossible to relate them directly to the carbohydrate fermented. A careful balance between the total products formed, the sugar fermented, and the ratios of these products to one another will

[3] Gayon, U., and Dubourg, E., *Ann. Inst. Pasteur*, 1894, viii, 108; 1901, xv, 527. Laborde, J., *Compt. rend. Acad.*, 1904, cxxxviii, 228. Müller-Thurgau, H., *Landw. Jahrb. Schweiz*, 1907, xxi, 230. Müller-Thurgau, H., and Osterwalder, A., *Centr. Bakteriol., 2te Abt.*, 1912, xxxvi, 129; 1917–18, xlviii, 1. Harden, A., *J. Chem. Soc.*, 1901, lxxix, 610. Kayser, E., *Ann. Inst. Pasteur*, 1894, viii, 737.

[4] Neuberg, C., and Reinfurth, E., *Biochem. Z.*, 1918, lxxxix, 365; xcii, 234. Neuberg, C., and Hirsch, J., *Biochem. Z.*, 1919, xcvi, 175.

frequently disclose relations that were unsuspected and help to give a more correct picture of the mechanism of the fermentation.

The most comprehensive chemical data on the products formed by organisms similar to *Lactobacillus pentoaceticus* are those of Gayon and Dubourg. They give complete data for the products formed from fructose, glucose, galactose, sucrose, maltose, lactose, and raffinose, and nearly complete data for mannose, sorbose, and xylose.

From glucose, galactose, and mannose they obtained acetic acid, lactic acid, alcohol, carbon dioxide, glycerol, and succinic acid. The proportion of these products varied somewhat with the age of the culture, the kind and amount of nutrients used, and the reaction of the medium. With the exception of glycerol the percentage of sugar represented by the different products falls in the neighborhood of that formed by the pentose fermenters. Their organism fermented raffinose but failed to attack arabinose, mannitol, glycerol, or malic acid, which is just the reverse of what takes place with the pentose fermenters of the group *Lactobacillus pentoaceticus*.

Müller-Thurgau and Osterwalder describe several mannite-forming bacteria which produced acetic acid, lactic acid, and alcohol from glucose and galactose. The relation of these products to one another varied with the organism concerned. Their papers give no data for the amount of gas formed or for the percentage of sugar represented by the end-products. A careful comparison of the pentose fermenters with their organisms shows many points of similarity, and also many differences in respect to fermentation power and ratio of products formed. The most marked differences are the fermentability of mannitol and lactates and the non-fermentability of raffinose by *Lactobacillus pentoaceticus*. Other differences in behavior manifest themselves toward arabinose, xylose, glycerol, and malates. Two of their organisms show high acetic acid production while a third, *Bacterium intermedium*, is more like *Lactobacillus pentoaceticus* in this respect. However, *Bacterium intermedium* ferments raffinose and fails to ferment arabinose, glycerol, and lactic acid.

The fermentation of glucose by *Bacillus coli communis* results in many of the same products and in quantities comparable to those formed by the pentose fermenters. From the data given

by Harden, acetic acid, lactic acid, alcohol, and carbon dioxide comprise the major products with small amounts or traces of succinic acid and formic acid. On the basis of the quantitative data obtained, Harden proposes the equation

$$2\ C_6H_{12}O_6 + H_2O = 2\ C_3H_6O_3 + C_2H_4O_2 + C_2H_6O + 2\ CO_2 + 2\ H_2$$

The most conspicuous difference between the products formed from glucose by Harden's *Bacillus coli communis* and by *Lactobacillus pentoaceticus* is the very small amount of acetic acid produced from glucose by the latter. As yet no evidence has been obtained with respect to the production of hydrogen by the pentose fermenters.

Kayser[5] made an extensive investigation of the conditions surrounding the production of lactic acid by bacteria, isolated from various materials. One of these, obtained from sauerkraut, possessed the power to ferment a large number of carbohydrates including many of the less common sugars such as xylose, arabinose, melezitose, trehalose, and raffinose. His paper gives no data for the quantity of products formed other than volatile acid, mainly acetic, and lactic acid.

In a later paper Kayser[6] gives quantitative data for the production of acetic acid, lactic acid, ethyl alcohol, carbon dioxide, and mannitol for a number of sugars. These bacteria differ from the pentose fermenters with respect to the kind of volatile acids formed and the ratios of the various end-products.

EXPERIMENTAL.

Fermentation.—The culture solution for fermentation was prepared by dissolving the glucose in a yeast water extract and sterilizing for 30 minutes at 15 pounds pressure. After sterilization, the solution was inoculated, and in certain cases sterilized calcium carbonate added. When calcium carbonate was not used, the acidity which developed during fermentation as shown by the presence of bromocresol purple was neutralized from time to time with sterilized 1 N NaOH.

[5] Kayser, E., *Ann. Inst. Pasteur*, 1894, viii, 737.

[6] Kayser, E., *Ann. Inst. nat. agron., Series 2*, 1904, iii, 241.

Volatile and Non-Volatile Acids.—At the end of the fermentation period the cultures were made up to a definite volume, centrifugalized to throw down the calcium carbonate, and an aliquot was taken for analysis. The volatile and non-volatile acids were determined by steam distillation and ether extraction, respectively, as previously described. The nature of the volatile acids formed was subsequently determined by subjecting them to a Duclaux distillation and by the barium content of their barium salts. The volatile acid proved to be almost entirely acetic.

The non-volatile acids extracted by the ether were determined by the Moeslinger method[7] for lactic acid and their identity was established by the zinc salt. The non-volatile acid was found to be racemic lactic acid. No evidence of any other non-volatile acid has been secured.

Alcohol.—The alcohol was obtained by saturating the neutralized culture solution with sodium chloride and then distilling over one-half to two-thirds of the aliquot taken. The distillate was oxidized with potassium dichromate and sulfuric acid as described by Dox and Lamb,[8] and then distilled. The acids thus obtained were identified in the same manner as already described for the volatile acids.

Carbon Dioxide.—When carbon dioxide and alcohol were determined, the fermentation was carried on in the apparatus described in a former paper[2] and the absorbed carbon dioxide determined by means of the Van Slyke[9] apparatus for measuring gases.

Fermentation of Glucose in the Presence of Calcium Carbonate.

Fermentation took place readily, but the analytical data showed only small amounts of acetic acid and relatively large amounts of lactic acid. Differences in the ratios between these two acids and data collected from the fermentation of fructose indicated that in old cultures the lactic acid in part was converted into acetic. Large cultures were therefore set up and aliquots were removed and analyzed at different periods in the fermentation.

[7] Moeslinger, Z. *Untersuch. Nahrungs-u. Genussmittel*, 1901, iv, 1120.
[8] Dox, A. W., and Lamb, A. R., *J. Am. Chem. Soc.*, 1916, xxxviii, 2561.
[9] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Marked increase in the quantity of acetic acid was found while the lactic acid remained stationary or decreased in amount. In an old culture the formation of lactic acid from glucose proceeds at about the same rate as the decomposition of lactic acid into acetic acid. Hence the latter accumulates while the quantity of the former remains stationary or decreases. In the case of Culture 41–11, the acetic has increased from 0.087 to 0.416 gm. per 100 cc., while the lactic remained practically unchanged at 0.717 gm. With Culture 118–8, the increase for acetic is from 0.078 to 0.328 gm., while the lactic shows an actual decrease from 0.713 to 0.637 gm. The data are given in Table I.

TABLE I.

Fermentation of Glucose in the Presence of Calcium Carbonate for Various Lengths of Time. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Weight of glucose.	Volatile acid as acetic.	Non-volatile acid as lactic.	Ratio acetic:lactic.
	days	*gm.*	*gm.*	*gm.*	
41–11	15*	2.00	0.087	0.717	100:824
41–11	30	2.00	0.157	0.722	100:460
41–11	75	2.00	0.416	0.720	100:173
118–8	15*	2.00	0.078	0.713	100:914
118–8	30	2.00	0.112	0.740	100:661
118–8	75	2.00	0.328	0.637	100:194

* Both cultures analyzed at successive intervals during the fermentation.

Attention is called to similar results obtained by Kayser, and Gayon and Dubourg. The former reports almost complete destruction of the lactic acid at the end of 20 days. Gayon and Dubourg found 1.62 gm. of acetic acid and 6.10 gm. of lactic acid in a 9 day culture, and 25 days later found the acetic acid had increased to 3.73 gm., while the lactic acid had decreased to 4.68 gm.

Loss of Alcohol from Fermenting Cultures.

Duplicates of the cultures whose volatile acid content is given in Table I were examined for ethyl alcohol, but the results were either negative or inconclusive. This failure to find alcohol proved to be due to evaporation from the fermentation flask.

The culture solution of 50 cc. volume was contained in a 300 cc. Erlenmeyer flask, stoppered in the usual way with a cotton plug, and after 32 days of fermentation had evaporated to about 40 cc.

Because of the small amount of alcohol found, a second experiment was set up where 500 cc. portions of solution were fermented in liter flasks. At the end of the fermentation period (23 days), the alcohol in the entire culture was distilled out and concentrated to a volume of 200 cc. The alcohol in 50 cc. was determined by the dichromate oxidation method while another portion (100 cc.) was concentrated to 33 cc. by successive distillations. This solution contained sufficient alcohol to give a good burning test and the characteristic iodoform crystals. These results indicate that great loss of volatile products such as alcohol, esters, and possibly acids may occur when flasks are stoppered with

TABLE II.

Loss of Alcohol from Flasks Stoppered with Cotton Plugs.

Culture No.	Age of culture.	Weight of sugar.	Volume of fermenting solution.	Volume of flask.	Ethyl alcohol.	Sugar represented by alcohol.
	days	*gm.*	*cc.*	*cc.*	*gm.*	*per cent*
55–9	32	1.00	50	300	0.017	1.7
69–19	32	1.00	50	300	0.010	1.0
41–11	23	20.00	500	1,000	2.301	11.5
118–8	23	20.00	500	1,000	2.608	13.0

loose cotton plugs. In this connection it is of interest to note the loss of acetone from cultures of *Bacillus acetoethylicum* recently reported by Northrop, Ashe, and Senior.[10] The data involved in these fermentations are given in Table II.

Balance of Products from the Fermentation of Glucose.

In these experiments all the products which had been shown to be formed were determined. Culture solutions of 250 to 400 cc. were fermented, and at the end of the fermentation period the various products formed were determined on different aliquots. At the same time the unfermented sugar was determined. The

[10] Northrop, J. H., Ashe, L. H., and Senior, J. K., *J. Biol. Chem.*, 1919, xxxix, 5.

data are given in Table III and bring out some very interesting relations.

1. In general, not more than 70 to 80 per cent of the glucose is fermented. The single exception to this is the 25 day Culture 118–8. The fermentation in the case of the aldo-hexoses is much less complete than in that of the pentoses. The inhibiting factor must be something other than the salts of the acids produced, for the concentration of these is higher with the pentoses than with the aldo-hexoses. No explanation can be given at present for this incomplete fermentation.

2. The sum of the four products determined represent from 85 to 95 per cent of the sugar fermented. The unknown 5 to 15 per cent would include any inaccuracies in the analytical methods,

TABLE III.

Total Fermentation Products from Glucose. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Weight of glucose fermented.	Acetic acid.	Lactic acid.	Ethyl alcohol.	Carbon dioxide.	Glucose accounted for by products.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
41–11	6	0.80	0.035	0.254	0.156	0.150	74.3
41–11	14	1.22	0.051	0.474	0.248	0.293	87.6
41–11	20	1.05	0.070	0.343	0.197	0.251	82.0
118–8	25	1.92	0.092	0.816	0.440	0.503	96.4

the sugar incorporated in the cells of the organisms, and minor products that have hitherto escaped detection.

3. The ethyl alcohol and carbon dioxide are produced in nearly equivalent quantities. The slight excess of carbon dioxide over ethyl alcohol may be due in part to a secondary fermentation of lactic acid as will be shown later. The nearly equal quantities of alcohol and carbon dioxide produced by many bacteria have been recorded by different investigators. This observation suggests the possibility that alcohol and carbon dioxide are formed from intermediate products similar in character to those found in yeast fermentation.

From the data given in Table III the relation of the different products to one another has been calculated and expressed as percentage of the total products formed. The figures thus ob-

tained are compared with the percentage relations that should exist if the fermentation proceeded according to the theory already proposed. An examination of the data given in Table IV shows values too low for lactic acid, and too high for carbon dioxide. The presence of 5 to 8 per cent of acetic acid is not allowed for in the theoretical equation. Very good agreement exists between the found and the calculated values for alcohol. The 5 to 8 per cent of acetic acid and excess 3 per cent of carbon dioxide are roughly equal to the deficiency in the value for lactic acid. The decreased ratio of acetic acid to lactic acid in old cultures, and the direct fermentation of lactic acid into acetic acid and carbon dioxide seem to warrant this explanation. The data are given in Table IV.

TABLE IV.

Percentage Distribution of Fermentation Products from Glucose.

Chemical compound.	Culture 41-11.			Culture 118-8.	Average.	Theory.
	6 days.	14 days.	20 days.	25 days.		
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Acetic acid.....	5.9	5.0	8.1	5.0	6.0	0.0
Lactic "	42.7	44.3	40.0	44.1	42.8	50.0
Alcohol.....	26.3	23.2	22.9	23.8	24.0	25.6
Carbon dioxide.....	25.2	27.4	29.1	27.2	27.2	24.4

Fermentation of Galactose.

The products obtained by the fermentation of galactose are the same as those obtained from glucose, but more galactose seems to have been consumed by the bacteria than was the case with glucose. The evidence on this point is somewhat conflicting and does not warrant any positive statement. The organisms seem to use these two sugars with equal ease. The total products formed and the percentage relations of the different products are given in Tables V and VI.

TABLE V.

Total Fermentation Products from Galactose. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Weight of sugar fermented.	Acetic acid.	Lactic acid.	Ethyl alcohol.	Carbon dioxide.	Galactose accounted for by products.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
41–11	33	1.406	0.086	0.522	0.389	0.343	95.3
118–8	10	1.385	0.064	0.475	0.341	0.305	85.5
118–8	33	1.542	0.065	0.545	0.436	0.409	94.4

TABLE VI.

Percentage Distribution of Fermentation Products from Galactose.

Chemical compound.	Culture 41–11.	Culture 118–8.		Average of three cultures.
	per cent	*per cent*	*per cent*	*per cent*
Acetic acid	6.4	5.4	4.5	5.4
Lactic "	39.0	40.1	37.4	38.8
Ethyl alcohol	29.0	28.8	30.0	29.3
Carbon dioxide	25.6	25.7	28.1	26.5

TABLE VII.

Total Fermentation Products from Mannose. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Weight of sugar fermented.	Acetic acid.	Lactic acid.	Ethyl alcohol.	Carbon dioxide.	Mannose accounted for by products.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
41–11	37		0.328	0.324			
41–11	16	0.853	0.097	0.259	0.182	0.191	85.5
118–8	38	0.578	0.084	0.104	0.141	0.173	87.0

TABLE VIII.

Percentage Distribution of Fermentation Products from Mannose.

Chemical compound.	Culture 41–11.	Culture 118–8.
	per cent	*per cent*
Acetic acid	13.3	16.7
Lactic "	35.5	20.8
Ethyl alcohol	25.0	28.0
Carbon dioxide	26.2	34.5

Fermentation of Mannose.

Mannose is generally regarded as a sugar that cannot be easily fermented by lactic acid-producing bacteria, and the results obtained with *Lactobacillus pentoaceticus* agree with this general experience. Only from 30 to 45 per cent of the sugar is consumed after 38 days. The difficulty with which mannose is fermented is probably related to the stereoisomeric configuration of mannose. Its configuration is more unlike the *d*-glucose type of structure than any of the other sugars used; *viz.*, glucose, galactose, xylose, or arabinose. The data for the fermentation products formed are given in Table VII.

The percentage distribution of the various products formed from mannose is given in Table VIII, and reveals a higher production of acetic acid and carbon dioxide, and a lower production of lactic acid than that obtained from either of the other two sugars. The difficult fermentation of mannose evidently forces the bacteria to destroy more lactic acid than is the case with the more readily fermented glucose and galactose.

Fermentation of Lactates.

As has already been pointed out in connection with the fermentation of fructose, mannitol, and glucose, there was an enormous change in the ratio of acetic acid to lactic acid in cultures where fermentation was continued for 50 to 70 days. In some cases there was an actual decrease in the quantity of lactic acid. This could only mean that some of the lactic acid was destroyed in the later stages of the fermentation, and possibly acetic acid was formed as one of the decomposition products. An attempt was therefore made to ferment the lactate directly. In the first experiment 2 gm. of sodium lactate syrup were added to the usual yeast water and inoculated with the pentose fermenters. Growth was apparent in 1 or 2 days; the medium became turbid, the bromocresol purple present indicated a change in reaction, and stained mounts showed the presence of large numbers of bacteria. After 17 days the control and two of the cultures were analyzed for volatile acid and unfermented lactic acid. It was found in the case of Culture 118–8 that the volatile acid had increased from

4.7 cc. of 0.1 N acid in the control to 28.3 cc. in the culture, and the lactic acid had decreased from 113.0 cc. of 0.1 N acid in the control to 91.0 cc. in the culture. The total acidity had remained practically unchanged, being 117.7 cc. of 0.1 N acid for the control and 119.3 cc. in the fermented solution. The volatile acid formed was later identified as acetic acid. After 37 days of fermentation two other cultures of the same series were analyzed and showed a further increase in acetic acid and a corresponding decrease in lactic acid.

TABLE IX.

Fermentation of Sodium Lactate. Calculated for 100 Cc. of Culture.

Culture No.	Age of culture.	Acetic acid.	Lactic acid.	Carbon dioxide.	Ratio acetic: CO_2
Series I.	*days*	*gm.*	*gm.*	*gm*	
Control.	17	0.028	1.016		
41–11	17	0.152	Lost.		
118–8	17	0.170	0.818		
41–11	37	0.226	0.725		
118–8	37	0.242	0.689		
Series II.					
Control.	48	0.026	Lost.		
41–11	48	0.074	1.043	0.034	100:71*
118–8	48	0.077	1.031	0.031	100:61*

* The value of the acetic acid control was deducted before calculating the ratios.

In the second experiment a carbon dioxide absorption bottle was attached to each fermentation flask and the absorbed gas determined at the end of the fermentation period. As judged by the quantity of acetic acid formed, the lactic acid was not decomposed to the same extent as was the case in the first experiment. The ratio of acetic acid to carbon dioxide was calculated and approximates that required for the theory that a molecule of carbon dioxide is formed for each molecule of acetic acid. The complete data calculated in gm. per 100 cc. of culture are given in Table IX.

Identification of Products.

Volatile Acid.—After titrating the volatile acids with 0.1 N Ba $(OH)_2$ the distillate was evaporated to dryness, taken up with about 60 to 70 cc. of hot water, and filtered into 300 cc. Erlenmeyer flasks. The flask was heated to boiling and when cooled was closed with a one-hole rubber stopper containing a carbon filter funnel. The end of the funnel was drawn out to a fine

TABLE X.

Distilling Constants of the Volatile Acids Obtained by the Duclaux Method.

Source of volatile acid.	Culture No.	Fractions.									
		10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
Glucose.	41–11*	7.5	15.4	23.7	32.5	41.6	51.1	61.1	72.3	84.8	100
"	41–11†	7.7	15.6	24.1	32.9	42.1	51.7	62.4	73.1	85.5	100
Alcohol from glucose.	41–11	7.1	15.6	24.0	32.8	42.0	51.6	61.9	73.0	85.0	100
Galactose.	118–8	7.9	16.1	24.6	32.3	42.3	51.8	61.9	72.9	85.2	100
Alcohol from galactose.	41–11	7.6	15.6	24.0	32.4	41.8	51.4	61.6	72.7	85.1	100
Mannose.	118–8	8.1	16.4	25.0	33.9	43.1	52.6	62.6	73.5	85.7	100
Alcohol from mannose.	118–8	8.3	16.6	25.0	33.8	42.9	52.5	62.6	73.5	85.7	100
Lactic acid.	41–11	7.8	15.8	24.3	33.1	42.3	51.9	62.2	73.2	85.5	100
Duclaux distilling constant for acetic acid.		7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100

* 20 day culture.
† 75 " "

capillary tube. The amount of 1 N H_2SO_4 required to decompose the barium salts was then added through the dropping funnel. No volatile acids could thus be lost from the hot solution. After standing a few hours at room temperature, the barium sulfate was filtered off, the filtrate made up to 110 cc., and subjected to Duclaux distillation. In the case of alcohol, the acid formed by oxidation and subsequent distillation was put through the same manipulation. The distilling constants obtained are given in Table X and agree with that found for acetic acid. In the case

of mannose, a small quantity of higher acid and alcohol seems to be present. An exact check cannot be expected as the controls always give 3 to 4 cc. of 0.1 N volatile acid. This acid originates either from the yeast water or from the sugars as a result of sterilization. It is also apparent that the alcohol formed in the fermentation is ethyl alcohol.

TABLE XI.

Composition of the Barium Salts of the Volatile Acid.

Culture No.	Compound fermented.	Barium salt of the volatile acid.	Barium sulfate equivalent.	
			Found.	Calculated for acetic acid.
		gm.	*gm.*	*gm.*
41–11	Glucose.	0.4248	0.3830	0.3957
41–11	Alcohol from glucose.	0.4106	0.3722	0.3825
41–11	Galactose.	0.2170	0.1900	0.1973
41–11	Alcohol from galactose.	0.9796	0.8912	0.8951
118–8	Mannose.	0.2056	0.1840	0.1879
118–8	Alcohol from mannose.	0.3406	0.3068	0.3112
41–11	Lactic acid.	0.4418	0.3958	0.4036

TABLE XII.

Water of Crystallization Contained in Zinc Lactate.

Culture No.	Source of salt.	Weight of zinc lactate used.	Water lost.		Water in $Zn(C_3H_5O_3)_2 + 3H_2O$.
		gm.	*gm.*	*per cent*	*per cent*
41–11*	Glucose.	0.4086	0.0696	17.0	18.17
41–11†	"	0.4414	0.0772	17.5	18.17
118–8	Galactose.	0.7126	0.1284	18.0	18.17
118–8	Mannose.	0.1670	0.0288	17.3	18.17

* 20 day culture.
† 75 " "

As a check on the Duclaux distilling constants, the barium content of the volatile acids has also been determined. The data found are given in Table XI and furnish evidence that the volatile acid is acetic, and the alcohol is ethyl.

The Form of Lactic Acid Produced.—The zinc salt was prepared from the barium salt of the lactic acid formed and the water of

crystallization determined. As is well known, the inactive zinc lactate crystallizes with 3 molecules of water, 18.17 per cent. From 17.0 to 18.0 per cent of water was found which indicates that the lactic acid formed is of the racemic type. The appearance of the crystals under the microscope resembled that of *d-l*-lactic acid. The data are given in Table XII.

SUMMARY.

The aldo-hexoses, glucose, galactose, and mannose, are fermented by *Lactobacillus pentoaceticus*, with the production of lactic acid, ethyl alcohol, carbon dioxide, and small quantities of acetic acid.

The acetic acid is probably not a direct fermentation product, but results from a secondary fermentation of the primary product, lactic acid. Evidence for this statement is based on the changing ratio of acetic to lactic with the age of the culture. In the early stages of the fermentation this is about 100:800, while in old cultures, 75 days, this ratio has decreased to 100:200. Not only has the ratio changed, but there has been an actual loss of lactic acid from the fermenting solution.

Further proof of the fermentation of lactic acid to acetic is adduced from the direct fermentation of lactates. The products formed are acetic acid and carbon dioxide.

Glucose and galactose are fermented at approximately the same rate and to the same extent. Mannose is more slowly attacked and less of the sugar is consumed. The unfermented glucose and galactose remaining after 30 to 40 days is about 20 to 30 per cent. In the case of mannose, the amount is from 50 to 70 per cent. The difficult fermentation of mannose results in a corresponding increase in the secondary fermentation of lactic acid to acetic acid.

The difference in the extent of fermentation of glucose, galactose, and mannose must rest on their configuration. The difference in stereoisomerism between an aldose and a ketose as illustrated by glucose and fructose results in the production of ethyl alcohol as one of the major products from glucose, and of acetic acid from fructose. This result is probably due to different conditions of oxidation and reduction operating on the intermediate products of the fermentation.

THE METABOLISM OF SULFUR.

III. THE RELATION BETWEEN THE CYSTINE CONTENT OF PROTEINS AND THEIR EFFICIENCY IN THE MAINTENANCE OF NITROGENOUS EQUILIBRIUM IN DOGS.

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(Received for publication, April 5, 1920.)

The importance of cystine for normal growth of the young white rat has been demonstrated by Osborne and Mendel (1) and more recently by Johns and Finks (2). In consequence of this work cystine is generally recognized as one of the essential amino-acids which must be present in adequate amounts in the diet. In acceptance of this belief most workers in nutrition experiments with hydrolyzed proteins have added cystine to replace that destroyed in the hydrolysis, particularly in acid hydrolysis (3, 4). Concerning the requirement of cystine for maintenance of adult animals, less information is available. Geiling (4) concluded that cystine was necessary for the maintenance of adult white mice.

In a former paper (5), it has been shown that the addition of cystine in small amounts to the food of dogs maintained on a low protein level diminished the loss of nitrogen from the body and favorably influenced the nitrogen balance. Since the addition of tyrosine and glycocoll as typical essential and non-essential amino-acids respectively exerted no influence on the condition of nitrogenous equilibrium under the same experimental conditions, the effect of cystine was considered specific. The work indicated that cystine was the amino-acid which determined the

* Some of the preliminary work in these experiments was carried on with the aid of Mr. G. E. Simpson.

protein minimum for the mixture of proteins fed, *i.e.* beef heart, and furnished strong experimental evidence of the indispensability of preformed cystine in adequate amounts for the maintenance of adult animals. A more definite interpretation of the data was difficult since the cystine content of the mixed protein fed, the various proteins contained in the heart muscle of cattle and in the blood of the coronary vessels, was unknown. Analysis of the mixed proteins isolated from the material fed in the experiments showed a rather low content of sulfur (0.73 per cent).

It was considered desirable to extend the work to include proteins of known sulfur (and cystine) content in order to ascertain to what extent the minimum for these proteins for maintenance of species other than the white rat and mouse was determined by their cystine content. The present investigation was planned to answer as far as possible the following questions. Do pure proteins of known sulfur (and cystine) content vary in their efficiency for the maintenance of nitrogenous equilibrium under conditions of a low protein input according to the relative amounts of sulfur (and cystine) they furnish? Can a protein low in sulfur (and cystine) be made more adequate for nutrition under the same conditions by the addition of cystine, the only definitely proved sulfur-containing complex of the protein molecule?

Casein and serum proteins were chosen for the experimental study as representative cystine-poor and cystine-rich proteins respectively. Casein contains about 0.8 per cent sulfur, but it is probable that only a small part of this sulfur is present as cystine. Attempts to isolate cystine from the hydrolysis products of casein have met with little success. Mörner (6) was able to obtain only 17 mg. of cystine from 100 gm. of casein. Casein on treatment with strong alkali yields about 13 per cent of its sulfur as alkali sulfide (7), which on the assumption that all this sulfide sulfur originates from cystine corresponds to about 0.60 per cent of cystine (1). Serum albumin contains a higher percentage of sulfur than casein and yields about two-thirds of its sulfur as alkali sulfide on boiling with alkali (7). Mörner (6) obtained 1.2 per cent cystine on hydrolysis of a commercial preparation of dried serum proteins and a like amount from purified serum albumin. Inasmuch as the quantitative methods for the isolation of

cystine are very inadequate, these figures are presumably much too low.

Analyses of casein by the Van Slyke method for the distribution of nitrogen have yielded results at variance with one another. The analyses of Van Slyke (8) show 0.20 per cent of the total nitrogen as cystine nitrogen, corresponding to a cystine content of about 0.27 per cent. Hart and Sure obtained similar figures (9). On the other hand, Crowther and Raistrick (10) obtained an average of 1.30 per cent[1] of the total nitrogen present as cystine nitrogen, corresponding to 1.77 per cent cystine. Hartley (11) found 2.75 per cent cystine nitrogen, in percentage of total nitrogen, in the serum proteins of ox blood. This was equivalent to 3.78 per cent of cystine.

EXPERIMENTAL.

The methods and general plan of the experiments were similar to those outlined in a former paper (5). The relative efficiency in nutrition of serum albumin, casein, and casein plus cystine, under conditions of a low plane of protein intake, was determined by a comparison of the nitrogen balances of the various experimental periods. In the preliminary experiments, an attempt was made to utilize the pure proteins as the sole source of nitrogen, but the animals refused the food within a short time. In the studies recorded in Tables I to III, therefore, 15 to 20 gm. of beef heart (0.4 to 0.6 gm. of N) were included in the standard diets and the pure proteins added to this. After this change little difficulty was experienced in obtaining complete consumption of the daily ration. The nitrogen added in the form of the beef heart comprised about 20 to 25 per cent of the total nitrogen of the diet, and was constant throughout the experiment, the greater part of the nitrogen being furnished by the protein under investigation.

The casein was prepared according to the method of Hammarsten. The serum protein (Albumin aus Blut, Kahlbaum) was entirely soluble in water, but contained some protein precipitable

[1] Professor H. S. Grindley of the Division of Animal Nutrition of this University has informed the writer that the work in his laboratory on the analysis of casein by the Van Slyke method has given results similar to those obtained by Crowther and Raistrick (10).

TABLE I.

Dog A, Long Haired Female.

Standard diet: Lard, 50 gm.; cane sugar, 60 gm.; starch, 20 gm.; beef heart, 15 gm.; calcium phosphate, 10 gm.; water, 400 cc.

Period.	Day.	Weight.	Intake nitrogen.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	
	1	11.16	1.81	2.13	0.27	−0.59	Standard diet and 10 gm. of casein.
	2	11.13	1.81	2.34	0.27	−0.80	
I	3	11.13	1.81	2.27	0.27	−0.73	
	4	11.13	1.81	2.24	0.27	−0.70	
	5	11.09	1.81	2.28	0.27	−0.74	
	6	11.09	1.81	2.15	0.27	−0.61	
Total			10.86	13.41	1.62	−4.17	
Average			1.81	2.24	0.27	−0.70	
	7	11.11	1.81	1.80	0.40	−0.39	Standard diet and 11.65 gm. of serum albumin.
	8	11.11	1.81	1.67	0.40	−0.26	
II	9	11.11	1.81	1.72	0.40	−0.31	
	10	11.11	1.81	1.73	0.40	−0.32	
	11	11.09	1.81	1.72	0.40	−0.31	
	12	11.09	1.81	1.72	0.40	−0.31	
Total			10.86	10.36	2.40	−1.90	
Average			1.81	1.73	0.40	−0.32	
	13	11.07	1.81	2.14	0.37	−0.70	Standard diet and 10 gm. of casein.
	14	11.09	1.81	2.26	0.37	−0.82	
III	15	11.07	1.81	2.32	0.37	−0.88	
	16	11.07	1.81	2.28	0.37	−0.84	
	17	11.05	1.81	2.28	0.37	−0.84	
	18	11.05	1.81	2.31	0.37	−0.87	
Total			10.86	13.59	2.22	−4.95	
Average			1.81	2.27	0.37	−0.83	
	19	11.09	1.81	1.45	0.30	+0.06	Standard diet, 9.36 gm. of casein, and 0.75 gm. of cystine.
	20	11.11	1.81	0.95	0.30	+0.56	
IV	21	11.13	1.81	1.13	0.30	+0.38	
	22	11.16	1.81	1.13	0.30	+0.38	
	23	11.16	1.81	1.23	0.30	+0.28	
	24	11.13	1.81	1.20	0.30	+0.31	
Total			10.86	7.09	1.80	+1.97	
Average			1.81	1.18	0.30	+0.33	

TABLE II

Dog G, Long Haired Female Collie.

Standard diet: Lard, 50 gm.; starch, 20 gm.; cane sugar, 70 gm.; beef heart, 15 gm.; bone ash, 10 gm.; water, 450 cc.

Period.	Day.	Weight.	Intake nitrogen.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	
I	1	13.30	1.77	3.13	0.32	−1.68	Standard diet and 10 gm. of casein.
	2	13.23	1.77	2.91	0.32	−1.46	
	3	13.25	1.77	2.67	0.32	−1.22	
	4	13.32	1.77	2.74	0.32	−1.29	
	5	13.32	1.77	2.68	0.32	−1.23	
	6	13.10	1.77	2.65	0.32	−1.20	
Total			10.62	16.78	1.92	−8.08	
Average			1.77	2.80	0.32	−1.35	
II	7	13.20	1.77	1.78	0.25	−0.26	Standard diet, 9.51 gm. of casein, and 0.75 gm. of cystine.
	8	13.20	1.77	1.36	0.25	+0.16	
	9	13.20	1.77	1.42	0.25	+0.10	
	10	13.17	1.77	1.57	0.25	−0.05	
	11	13.18	1.77	1.62	0.25	−0.10	
	12	13.18	1.77	1.76	0.25	−0.24	
Total			10.62	9.51	1.50	−0.39	
Average			1.77	1.59	0.25	−0.07	
III	13	13.16	1.77	2.40	0.21	−0.84	Standard diet, 9.51 gm. of casein, and 0.47 gm. of glycocoll.
	14	13.16	1.77	2.82	0.21	−1.26	
	15	13.14	1.77	2.84	0.21	−1.28	
	16	13.11	1.77	2.57	0.21	−1.01	
	17	13.11	1.77	2.57	0.21	−1.01	
	18	13.07	1.77	2.36	0.21	−0.80	
Total			10.62	15.56	1.26	−6.20	
Average			1.77	2.59	0.21	−1.03	
IV	19	13.07	1.77	2.43	0.34	−1.00	Standard diet, 9.15 gm. of casein, and 1.13 gm. of tyrosine.
	20	13.05	1.77	2.40	0.34	−0.97	
	21	13.00	1.77	2.29	0.34	−0.86	
	22	13.05	1.77	2.32	0.34	−0.89	
Total			7.08	9.44	1.36	−3.72	
Average			1.77	2.36	0.34	−0.93	
V	23	13.00	1.77	2.05	0.21	−0.49	Standard diet, 9.35 gm. of casein, and 0.75 gm. of cystine.
	24	13.11	1.77	1.36	0.21	+0.20	
	25	13.16	1.77	0.99	0.21	+0.57	
	26	13.16	1.77	1.09	0.21	+0.47	
	27	13.19	1.77	1.43	0.21	+0.13	
Total			8.85	6.92	1.05	+0.88	
Average			1.77	1.38	0.21	+0.18	

TABLE III.

Dog A, Long Haired Female; Weight 12.0 Kg.

Standard diet: Lard, 50 gm.; starch, 40 gm.; cane sugar, 60 gm.; beef heart, 20 gm.; calcium phosphate, 10 gm.; water, 400 cc.

Period.	Day.	Intake nitrogen.	Urinary nitrogen.	Fecal nitrogen.	Nitrogen balance.	Diet.
		gm.	*gm.*	*gm.*	*gm.*	
	1	2.04	2.14	0.30	−0.40	Standard diet and 10 gm. of casein.
	2	2.04	2.30	0.30	−0.56	
	3	2.04	2.25	0.30	−0.51	
I	4	2.04	2.26	0.30	−0.52	
	5	2.04	2.29	0.30	−0.55	
	6	2.04	2.31	0.30	−0.57	
	7	2.04	2.22	0.30	−0.48	
Total		14.28	15.77	2.10	−3.59	
Average ..		2.04	2.25	0.30	−0.51	
	8	2.04	1.70	0.32	+0.02	Standard diet and 12.4 gm. of serum albumin.
	9	2.04	1.51	0.32	+0.21	
	10	2.04	1.53	0.32	+0.19	
II	11	2.04	1.58	0.32	+0.14	
	12	2.04	1.52	0.32	+0.20	
	13	2.04	1.83	0.32	−0.11	
	14	2.04	1.75	0.32	−0.03	
Total		14.28	11.42	2.24	+0.62	
Average ..		2.04	1.63	0.32	+0.09	
	15	2.04	1.92	0.34	−0.22	Standard diet and 10 gm. of casein.
	16	2.04	2.21	0.34	−0.51	
	17	2.04	2.07	0.34	−0.37	
III	18	2.04	2.13	0.34	−0.43	
	19	2.04	2.09	0.34	−0.39	
	20	2.04	1.92	0.34	−0.22	
	21	2.04	1.94	0.34	−0.24	
Total.		14.28	14.28	2.38	−2.38	
Average ..		2.04	2.04	0.34	−0.34	
	22	2.13	1.52	0.28	+0.33	Standard diet, 10 gm. of casein, and 0.75 gm. of cystine.
	23	2.13	1.31	0.28	+0.54	
	24	2.13	1.39	0.28	+0.46	
IV	25	2.13	1.36	0.28	+0.49	
	26	2.13	1.37	0.28	+0.48	
	27*	2.13	1.51	0.28	+0.34	
	28*	2.13	1.68	0.28	+0.17	
Total		14.91	10.14	1.96	+2.81	
Average ..		2.13	1.45	0.28	+0.40	

* Small amount of food not eaten.

by saturation with NaCl in neutral reaction, as well as some inorganic salts. In a later experiment (not recorded in this paper) in connection with other experiments, the protein was dissolved in water, coagulated by heat, and washed with hot water until tests for chlorides in the wash water were negative. The coagulated protein was then dried *in vacuo* and ground to a fine powder. The results with this more highly purified preparation were similar in every respect to those recorded here for the uncoagulated protein.

As shown in Tables I and III, maintenance of nitrogenous equilibrium was possible under conditions of a lower protein intake when the serum proteins, relatively high in cystine content, were the main source of the protein of the diet than when casein, low in its content of cystine, furnished the bulk of the protein. Thus on a nitrogen intake of 1.81 gm. daily (Table I), the average nitrogen balances were −0.70 and −0.83 gm. respectively during the fore and after periods when casein was fed, as compared with a balance of −0.32 gm. in the serum protein period. With a higher nitrogen intake of 2.04 gm. per day (Table III), the average daily nitrogen balances were −0.51 and −0.34 gm. respectively with casein, and +0.09 gm. with serum albumin. The serum proteins were also more efficient in the maintenance of nitrogenous equilibrium than were the proteins of beef heart when the latter constituted the sole source of protein in the diet (5).

When casein was supplemented by the addition of cystine to the diet (Table I, Period IV; Table II, Periods II and V; Table III, Period IV), the balance of nitrogen became positive in every case except one. The failure of glycocoll and tyrosine to influence the balance of nitrogen (Table II) is additional proof of the specific demand for cystine on a diet poor in this amino-acid. These results are in harmony with the work of Osborne and Mendel (1), in which these observers reported that growth in white rats occurred at a lower plane of protein intake when casein was supplemented by cystine than with casein alone. In the present series of experiments, casein supplemented by cystine was apparently somewhat superior in nutritive value to serum albumin. This may possibly be explained by the fact that some amino-acid other than cystine is the limiting factor for this protein.

SUMMARY.

The relative efficiencies in nutrition of casein, a protein low in cystine content, and of serum albumin, high in cystine content, have been studied in dogs. Serum albumin under conditions of a low protein intake is more effective in maintaining nitrogenous equilibrium than is casein. When casein is supplemented by cystine, however, it is as efficient for the maintenance of nitrogenous equilibrium as is serum albumin. These experiments furnish additional evidence that cystine is essential for maintenance as well as growth.

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A METHOD FOR THE DETERMINATION OF METHEMOGOBLIN AND HEMOGLOBIN IN BLOOD.

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(Received for publication, April 5, 1920.)

While studying a type of cyanosis due to change in the blood pigment, with the formation of methemoglobin, need arose for a simple and accurate quantitative method for methemoglobin. A further incentive was the belief that such a method would be of considerable value in hospital or clinical laboratories, for application in cases of severe methemoglobinemia, when the amount of methemoglobin in the blood is of value in determining the advisability of certain therapeutic procedures, such as transfusion.

The method to be described was suggested by the observation that in blood containing methemoglobin the hemoglobin determined colorimetrically was considerably greater than the hemoglobin calculated from the oxygen capacity.[1]

Stadie[2] has recently published a method for methemoglobin identical with ours except that the total hemoglobin is determined as cyanhemoglobin after converting the hemoglobin into methemoglobin.

Principle of the Method.

The method is based upon the fact that while oxygen, as is well known, is liberated from hemoglobin by potassium ferricyanide it is not liberated from methemoglobin even *in vacuo*. The total hemoglobin (hemoglobin + methemoglobin) is determined

[1] A preliminary note was published by McEllroy, W. S., *J. Am. Med. Assn.*, 1919, lxxiii, 1927. The method was reported before the American Society of Biological Chemists (McEllroy, W. S., *J. Biol. Chem.*, 1920, xli, p. xlvii).

[2] Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.

colorimetrically as methemoglobin, using potassium ferricyanide to convert the hemoglobin into methemoglobin. The oxygen capacity is determined by Van Slyke's method,[3] from which is calculated the hemoglobin only. The hemoglobin as calculated from the oxygen capacity is subtracted from the total hemoglobin determined colorimetrically as methemoglobin. The difference is the amount of methemoglobin.

Description of the Method.

The blood is drawn from an arm vein and oxalate used to prevent clotting. 1 cc. of the blood is transferred to a 50 cc. volumetric flask containing 20 cc. of distilled water. For this purpose an Ostwald pipette calibrated to contain 1 cc. is satisfactory. The blood is drawn up to the mark, allowed to drain into the flask, and the drop in the tip blown out. Distilled water is then drawn up into the pipette and discharged into the flask. This is repeated until the blood in the pipette and any adhering to the neck of the flask is washed down into the flask. 1 cc. of a 4 per cent solution of potassium ferricyanide is added and the flask agitated to insure mixing. After standing for 5 minutes it is diluted to the mark with water, mixed, and compared in the colorimeter with a standard solution of methemoglobin. The cup containing the standard is set at 10 or 15 mm. The result gives the total hemoglobin (hemoglobin + methemoglobin) in gm. per 100 cc. of blood.

The oxygen capacity is determined on a portion of the same blood. From this is calculated the hemoglobin content in gm. per 100 cc. The hemoglobin calculated from the oxygen capacity is subtracted from the total hemoglobin (hemoglobin + methemoglobin) and the difference is the amount of methemoglobin in gm. per 100 cc. of blood.

Preparation of the Standard.

The standard solution of methemoglobin having a known hemoglobin content is prepared from normal defibrinated or oxalated blood. Defibrinated ox blood is convenient. The

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.

hemoglobin content of the normal blood is determined from its oxygen capacity. 10 cc. of the blood are transferred to a 500 cc. volumetric flask containing 200 cc. of water and 10 cc. of a 4 per cent solution of potassium ferricyanide solution added. The flask is agitated and allowed to stand for 10 minutes. It is then diluted to the mark with water and mixed. This gives a standard solution of methemoglobin, the blood pigment value of which is known, for the determination of the total hemoglobin as described above. In case the standard is much stronger than the unknown, suitable dilution of the standard should be made until one approximating in concentration that of the unknown is obtained. Of course the degree of dilution of the standard must be known.

The method of calculation is best illustrated by means of an example.

Standard solution of methemoglobin = 18 gm. of hemoglobin per 100 cc. of blood.

Colorimetric determination of total pigment as methemoglobin = standard 10 mm. and unknown 11 mm.

Then $\frac{10}{11} \times 18 = 16.36$ gm. of total pigment per 100 cc.

Hemoglobin calculated from oxygen capacity = 15 gm. per 100 cc.

Therefore $16.36 - 15 = 1.36$ gm. of methemoglobin per 100 cc. of blood.

DISCUSSION.

The total hemoglobin (hemoglobin + methemoglobin) cannot be determined colorimetrically as carbon monoxide hemoglobin because the methemoglobin is not converted into carbon monoxide hemoglobin quantitatively. The methemoglobin, however, apparently may be converted into acid hematin by hydrochloric acid and the total hemoglobin determined as such. As the spectroscopic data on this point is still incomplete the results will be published later.

The color of the methemoglobin is very nearly identical with that of acid hematin and can be compared accurately colorimetrically. Although the methemoglobin and acid hematin approximately match in color they have very dissimilar colorimetric values.

CONCLUSIONS.

In blood containing methemoglobin the amount of methemoglobin and hemoglobin can be determined quickly and accurately by the method described.

VITAMINE STUDIES.

V. THE ANTISCORBUTIC PROPERTIES OF RAW BEEF.*

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(Received for publication, March 30, 1920.)

INTRODUCTION.

The rapidly accumulating literature, both in England and in this country, concerning the antiscorbutic properties of food materials lends considerable support to the view that we are dealing with a third "food hormone" which can be placed in the list of substances called vitamines. In fact, the recognition of this vitamine has become sufficiently general for Drummond,[1] following the nomenclature of McCollum and Kennedy,[2] to designate this vitamine as "water-soluble C."

The work described in this paper was initiated as a part of a larger cooperative problem under investigation at this institution; *i.e.*, the study of the effect of heat as employed in the ordinary methods of food preparation upon the vitamine content of some of our common food materials. Meat was chosen as the first food to be studied, owing to the fact that human experience had indicated that this food possessed antiscorbutic properties, and also because our facilities were unusually good for obtaining meats cooked in a variety of ways.

* Published with the approval of the Director as Paper No. 202 of the Journal Series of the Minnesota Experiment Station.

A preliminary statement of this work was published in *Science*, 1919, l, 184.

[1] Drummond, J. C., *Lancet*, 1918, ii, 482.

[2] McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 493.

Our findings with regard to the antiscorbutic properties of beef, which are described in this paper, were such that the original plan, involving the cooking of beef, was, of necessity, abandoned.

Lind[3] is the first observer, as far as we are aware, to ascribe antiscorbutic properties to meat. In his early studies of tropical diseases (1771) this writer made the interesting observation that a soup prepared from the flesh of the green turtle was curative in human scurvy.

About 120 years later Barlow[4] advocated the use of meat juice or meat gravy as an antiscorbutic food for infants, although he emphasized the superiority of uncooked meat over that which had been cooked.

Smith,[5] in an interesting article on the historical phases of antiscorbutic foods and drinks, cites the case of the Grinnell expeditions in which "a certain amount of fresh meat" failed to prevent scurvy on the first expedition, while scurvy was thought to have been prevented on the second expedition by occasional supplies of fresh meat.

More recently Stefánsson,[6] describing his experiences in the Arctic regions, states that fresh meat prevented and cured scurvy in his party.

Wiltshire,[7] on the other hand, reports observations on 132 cases of scurvy among Serbian soldiers whose daily diet had contained meat which had been frozen. This writer is of the opinion that fresh raw meat contains "a small amount of antiscorbutic vitamine" but that freezing and cooking "render meat practically useless."

Chick, Hume, and Skelton[8] were unable to note any beneficial effect when meat juice was fed to guinea pigs, while Pitz[9] is of the opinion that dried meat delayed the onset of scurvy symptoms in the same type of experimental animal, due to the better plane of protein intake. This work is open to criticism, however, due to the fact that milk was fed *ad libitum*. The antiscorbutic property of milk is undoubtedly proportional to the quantity ingested and also, as we[10] suggested in a previous paper, to the nature and quantity of the fresh feeds eaten by the cow.

In a recent publication Givens and McClugage[11] have substantiated our work showing that they were unable to prevent scurvy in guinea pigs and, furthermore, they found that it was impossible to prolong life on scorbutic

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[3] Lind, J., An essay on diseases incidental to Europeans in hot climates, 1771, 2nd edition, 206.

[4] Barlow, T., *Lancet*, 1894, ii, 1075.

[5] Smith, A. H., *Lancet*, 1918, ii, 813.

[6] Stefánsson, V., *J. Am. Med. Assn.*, 1918, lxxi, 1715.

[7] Wiltshire, H. W., *Lancet*, 1918, ii, 811.

[8] Chick, H., Hume, E. M., and Skelton, R. F., *Biochem. J.*, 1918, xii, 131.

[9] Pitz, W., *J. Biol. Chem.*, 1918, xxxvi, 439.

[10] Dutcher, R. A., Pierson, E. M., and Biester, A., *Science*, 1919, l, 184.

[11] Givens, M. H., and McClugage, H. B., *Science*, 1920, li, 273.

diets by the ingestion of dehydrated beef. These results are not in accord with those of Pitz,[9] the explanation of which, Givens and McClugage believe, lies in the amount of milk consumed by the guinea pigs in Pitz's experiment. This view is also in accord with our former statements;[10] that is, that the milk should always be fed quantitatively, for "the anti-scorbutic properties of milk are proportional to the amount of milk ingested."

EXPERIMENTAL.

Care of Animals.—The animals used in the experiments described in this paper were healthy young guinea pigs weighing between 150 and 350 gm. No guinea pigs were used until they had been under observation for several days during which they were fed a normal diet of oats, cabbage, carrots, and hay. Guinea pigs which did not grow normally were eliminated from the experiment. The animals were grouped according to weight, each experimental group being made up of one heavy guinea pig, one light guinea pig, and two which fell between the two extremes in regard to body weight. This was done in order that the groups should be comparable, one with the other, and to eliminate as far as possible the effect of age and size of the animals on the average length of time elapsing before the onset of the disease.

The animals were confined, singly, in cages containing removable metal trays which were cleaned once or twice a week and sprayed with a cresol preparation. Clean fresh sawdust was used as bedding to absorb moisture. Weight records were taken every 4 days before feeding and the animals were under observation at practically all hours of the day. Autopsies were performed in all cases soon after death and ankle joints and costochondral junctions were placed in Zenker's fluid, washed and carried through dilute alcohol, and finally preserved in 95 per cent alcohol for histological examination.

Beef Extract.—Lean beef freed from gristle and fat was finely ground, and weighed portions were mixed with water and allowed to stand in a cool place for 12 to 15 hours. The mixture was then subjected to pressure in a hand press and the liquid extract carefully removed. The dry meat pulp was then treated with water a second time and allowed to stand for 2 hours. After a second pressing a third extraction was made. The water extracts were combined and made to such a volume that 2 cc. represented

the water-extractable material from 1 gm. of beef. Fresh extracts were made every 3rd day and these were kept on ice until used. The beef was procured in the open market.

Experimental Diet.—As has been customary with many investigators in this field, oats were fed *ad libitum*. In order to improve the protein and salt content of the ration 25 cc. of autoclaved milk were given daily. The milk was autoclaved for 1 hour at 120°C. to eliminate any possibility of introducing the antiscorbutic vitamine in the milk. Little difficulty was experienced in getting the animals to take the milk quantitatively from dishes, provided water was withheld for a time before feeding. The beef extract was fed by hand by means of a large medicine dropper. In a few cases it was also necessary to feed the milk by hand, especially during the latter stages of the disease.

The groups of animals in Series I received the following diets:

Group I.	Basal diet (oats, water, 25 cc. of autoclaved milk).							
" II.	"	"	+ 10 cc. of beef extract.					
" III.	"	"	+ 20	"	"	"	"	
" IV.	"	"	+ 30	"	"	"	"	
" V.	"	"	+ 40	"	"	"	"	
" VI.	"	"	+ 5 gm.	"	chopped raw beef.			
" VII.	Oats and water only.							

The detailed results of the experimental work in Series I are shown in Table I.

Examination of the data in Table I shows that the presence of the meat extract had little effect in preventing the onset of scurvy symptoms or in delaying death from scurvy. When plotted, all the curves are of the same type irrespective of the amount of beef extract fed. The average length of time elapsing before the symptoms were noted was about 15 days, which is about the time usually expected on oats alone. The average length of life was about 26 days, which is also the time usually expected on a scorbutic type of diet. It was not considered necessary to feed the dry beef residues which remained after extraction for the reason that the water-soluble antiscorbutic substance could not have remained behind in appreciable quantities in view of the methods employed. The feeding experiment with the solid raw beef (Table I) substantiates our original opinion in this regard, for the ingestion of the chopped raw beef showed no beneficial effects.

Histological examinations were made of joints and costochondral junctions of representative animals in each group and, in every case, our clinical observations were substantiated by the histological findings. In general, our observations are in accord with other investigators in this field with regard to the symptomatology of scurvy in guinea pigs. We have felt that the face ache position has been somewhat overemphasized as far as symptomatology is concerned. To be sure we have noted it in a number of cases, but a large proportion of our scorbutic animals have never been found in this position. On the other hand, it is not uncommon to find normal guinea pigs sleeping in a manner which simulates the face ache position. Other symptoms, such as partial paralysis of the hind quarters, tenderness of joints, swelling of joints, etc., have been observed to occur with great regularity and it does not require much experience to be able to tell within a reasonable limit of error when the first symptoms of scurvy appear. We have also found, in addition, swollen joints (which harden into exostoses), fragility of bones, and loosening of teeth. Postmortem examination invariably reveals hemorrhagic conditions and extravasations in many of the tissues, especially in the muscles of the hind legs. Swollen costochondral junctions are usually present and these are often hemorrhagic.

At the termination of the experiments outlined in Table I we were forced to conclude that beef extract does not contain a water-soluble antiscorbutic substance in sufficient quantity to prevent scurvy in the guinea pig. We realized that meat extractives and milk were not normal ingredients of a guinea pig diet and it was felt that it was possible that these foods might possess properties which were actually deleterious; therefore the work was repeated feeding orange juice in the presence and in the absence of meat extract.

In the confirmatory work (Table II, Series II), the basal diet was identical with that of Series I with the exception that 20 cc. of pasteurized milk were fed instead of 25 cc. of autoclaved milk. This was done with the view of dispensing with the work of autoclaving the milk, for it is well known[12] that milk is so deficient in the antiscorbutic vitamine that the small quantity fed (20 cc.)

[12] Barnes, R. E., and Hume, E. M., *Lancet*, 1919, ii, 323.

TABLE I.

Series I.

Influence of Cold Water Extract of Raw Beef upon Scurvy in Guinea Pigs (2 Cc. from 1 Gm. of Beef). Basal Diet Consisted of Oats and Water ad Libitum and 25 Cc. of Autoclaved Milk.

Group and diet.	Animal No.	Initial weight.	Final weight.	Gain or loss.	Day on which first scurvy symptoms were noted.	Average time for group.	Length of life.	Average life for group.	Postmortem examination.	Remarks.
		gm.	*gm.*	*per cent*		*days*	*days*	*days*		
I	1	304	201	−33.9	10	11.7	19	21.7	Scurvy.	
Basal diet.	2	225	150	−33.3	7		23		"	
	3	280	160	−42.9	15		20		"	
	4	160	120	−25.0	15		25		"	
II	5	358	250	−30.2	23	18.2	28	25.2	" and pneumonia	
Basal diet + 10 cc.	6	223	210	− 5.8	13		23		"	
beef extract.	7	253	160	−36.8	22		25		"	
	8	189	160	−15.3	15		25		"	
III	9	345	240	−30.4	14	14.2	23	24.0	"	
Basal diet + 20 cc.	10	240	157	−34.5	17		26		"	
beef extract.	11	211	170	−19.5	13		25		"	
	12	174	130	−25.3	13		22		"	
IV	13	257	183	−28.8	15	14.6	25	25.6	" and pneumonia.	
Basal diet + 30 cc.	14	330	245	−25.8	15		31		"	
beef extract.	15	226	167	−26.1	14		21		"	

V	17	365	248	−32.1		18.6	30	26.0	Scurvy.	
Basal diet + 40 cc.	18	278	195	−29.9	17		25		"	
beef extract.	19	233	200	−14.2	13		22		" and pneumonia.	
	20	153	165	+ 7.8	26		27		"	Cured on orange juice; no histological evidence of scurvy.
VI	51	320	175	−45.3		20(?)	22	26.0	General inanition.	
Oats + 5 gm. chopped raw beef.	52	325	225	−31.8			24		Scurvy.	
	53	250	147	−41.2	20		30		General inanition.	No scurvy lesions.
	54	271	180	−34.6			28		Scurvy.	
VII	21	247	135	−45.3	20	17.5	25	26.0	"	
Oats and water.	22	183	120	−34.4	15		24		"	
	23	175	110	−37.1			29		"	

could not influence the course of the disease to an appreciable extent.

The diets fed in Series II were as follows:

Group VIII. Basal diet (oats, water, 20 cc. of pasteurized milk).
" IX. " " + 5 cc. of orange juice.
" X. " " + 5 " " " " + 10 cc. of meat extract.
" XI. " " + 10 " meat extract.

The details of the feeding experiment in Series II are summarized in Table II.

The data in Table II verify the results obtained in Series I; *i.e.*, that the presence of meat extract did not prevent the onset of scurvy. When orange juice was fed (Group IX) no scurvy developed, and the same is true for Group X which received orange juice and beef extract. When the orange juice was omitted (Group XI), the presence of meat extract had no effect so far as scurvy is concerned. It would appear, also, that the beef extract had no detrimental effect when fed in the presence of orange juice.

The length of life, in Series II, was prolonged beyond the usual period, the probable explanation depending upon the diet of the cow from which the milk was obtained rather than upon the presence of the meat extract or the fact that the milk was not autoclaved. When the same milk was autoclaved for 1 hour at 120°C. curves and data were obtained similar to those with the pasteurized milk, thereby eliminating the possibility of the presence of antiscorbutic vitamine in the milk. Our reasons for this view will be published in detail in a later paper.

It may be possible that the explanation for the antiscorbutic properties of the meat in the diet of Stefánsson's party[6] lies in the fact that the meat was obtained, almost entirely, from the seal and the bear and included not only muscle tissue but other tissues, such as the liver. It is well to emphasize at this point that too great care cannot be exercised in applying the results of experiments with guinea pigs and rats to problems in human nutrition. Recent work of Hess and Unger[13] indicates that the demand of infants for the fat-soluble vitamine is much less than

[13] Hess, A. F., and Unger, L. J., *J. Am. Med. Assn.*, 1920, lxxiv, 217.

TABLE III.

Series II.

Confirmatory Data Relative to the Influence of Beef Extract in the Presence of Orange Juice. Basal Diet Consisted of Oats and Water ad Libitum + 20 Cc. of Pasteurized Milk.

Group and diet.	Animal No.	Initial weight	Final weight	Gain or loss.	Day on which first scurvy symptoms were noted.	Average time for group	Length of life.	Average life for group.	Postmortem examination	Remarks.
		gm.	*gm.*	*per cent*		*days*	*days*	*days*		
VIII	24	311	220	−29.3	29	24.6		39.5		Changed to curative diet on 40th day.
Basal diet.	25	286	180	−37.1	26		40		Scurvy.	
	26	265	205	−22.6	19		39		"	
IX	28	365	360	− 1.4						Changed to another diet on 60th day.
Basal diet + 5	29	367	435	+18.5						" " " " " 60th "
cc. orange	30	320	393	+22.8						" " " " " 60th "
juice.	31	230	245	+ 6.5						" " " " " 60th "
X	32	265	333	+25.7						" " " " " 60th "
Basal diet + 5	33	235	360	+53.2						" " " " " 60th "
cc. orange	34	283	380	+34.3						" " " " " 60th "
juice + 10 cc.	35	312	305	− 2.2						" " " " " 60th "
beef extract.										
XI	37	295	294	− 0.3	25	31.5		54.6	Scurvy.	Choked to death on 28th day.
Basal diet + 10	38	280	175	−37.5	36		54		"	
cc. beef ex-	39	268	199	−25.7	29		41		"	
tract.	40	425	260	−38.8	36		69			Changed to another diet on 69th day.

that indicated by previous work with rats. Similar discrepancies will doubtless appear as vitamine work progresses, due to the varying physiological demands for specific vitamines by the different species of animals.

SUMMARY AND CONCLUSIONS.

Guinea pigs were fed diets of oats, water, and an amount of milk sufficient to improve the diet but insufficient to prevent scurvy. These animals developed scurvy and died. When water extracts of raw lean beef were fed representing 5, 10, 15, and 20 gm. of raw beef no differences could be noted in the time of onset of scurvy or in the length of life of the experimental animals. Orange juice, added to the basal diet, prevented scurvy, both in the presence and absence of meat extract. The excellent condition of the animals on the orange juice-beef extract diet shows conclusively that the poor condition of the animals on the beef extract diet was due to the absence of the antiscorbutic vitamine rather than to any deleterious property of the beef extract.

In conclusion, we wish to acknowledge the assistance of Dr. H. E. Robertson for his preparation and interpretation of some of our histological material, Dr. C. P. Fitch for assistance in some of the postmortem examinations, and Mr. O. Mydland for assistance in caring for the experimental animals.

CRITICAL STUDY OF METHODS FOR THE DETECTION OF METHYL ALCOHOL.

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(Received for publication, March 11, 1920.)

During the years 1918 and 1919, I have had occasion to examine over 700 human organs for alcohol. In addition to this, about 250 liquors of various descriptions were analyzed.

In the work with liquors, wines, whiskeys, and cordials, a very interesting and far reaching observation was made, particularly in connection with medicolegal work. It was found, when the liquors were tested in the customary manner, that is by oxidation of the alcohols to aldehydes and then applying the usual color reactions (milk-hydrochloric acid test, resorcin test, gallic acid test), that many liquors which contained no methyl alcohol at all gave very good positive reactions to one or all three of the above named tests. Then again, liquors which contained even as much as 10 per cent methyl alcohol would often give a milk-hydrochloric acid test no different from a good ethyl alcohol liquor; that is, the characteristic methyl alcohol reaction was entirely masked (28). The cause of these false and masked reactions was the presence in the oxidized products of the liquors of a number of substances such as acetone, acetaldehyde, ethyl alcohol, formic acid, dimethyl-ethyl-carbinol, secondary and tertiary butyl alcohol, essential oils, acrolein, and furfurol. The more examinations I made, the more I was impressed with the unreliability of the above tests. Therefore, it appeared of great importance to make a study of all the tests for methyl alcohol reported in the literature in order to find a set of tests which, when properly applied, would give results upon which there could be placed absolute reliance.

58 tests for methyl alcohol were found in the literature. All these were put through a critical study upon eighteen liquors of the following description:

Eight typical good grain alcohol whiskies.

One combined distillate of 58 liquors free from methyl alcohol and containing 42 per cent alcohol.[1]

One mixture of 5 per cent methyl alcohol plus 55 per cent of above combined distillate plus 40 per cent water.

One mixture of 10 per cent methyl alcohol plus 50 per cent of above combined distillate plus 40 per cent water.

One mixture of 20 per cent methyl alcohol plus 40 per cent of above combined distillate plus 40 per cent water.

One mixture of 30 per cent methyl alcohol plus 30 per cent of above combined distillate plus 40 per cent water.

Five typical methyl alcohol liquors (confiscated).

Each of these eighteen liquors was subjected to all the 58 tests. The reactions marked with an asterisk were not completely studied, either because the reagent was not obtainable or because the small amount on hand was not sufficient to permit the testing of all the eighteen samples. By applying each test to a series of liquors, as the one indicated above, practically all the characteristics, such as sensitiveness, reliablity, specificity, and influence upon the reaction of foreign substances, were brought out.

The tests for methyl alcohol can be classified into two groups: (*A*) That group in which the methyl alcohol must first be oxidized into formaldehyde before the tests are applied; (*B*) that group in which no oxidation is necessary, the test being applied directly to the alcohol molecule. In both groups of methods it is imperative, first, to distill fractionally the liquor, the object being to free the alcohols from non-volatile admixed substances[2] and, second, to obtain the alcohol in more concentrated form.

[1] The object of using the combined sample of so many liquors was to get as many as possible of the interfering substances that naturally occur in the average drinks.

[2] Some ciders, when oxidized directly, responded beautifully to all the color reactions for formaldehyde. If they were previously distilled and then oxidized, however, no such reactions were obtained. The physical tests and the tests depending on crystalline structure proved the absence of methyl alcohol.

Methods of Group A.

In this group the tests depend upon the reaction of formaldehyde with the various reagents mentioned below. It is therefore necessary first to convert the methyl alcohol to formaldehyde. This is brought about by oxidation. Several methods for oxidizing methyl alcohol into formaldehyde are available:

(*a*) Plunging a red hot copper spiral into the liquid.

(*b*) Passing the vapors of alcohol mixed with air through a tube over hot copper.

(*c*) By finely divided platinum.

(*d*) Alkaline permanganate.

(*e*) Potassium dichromate and sulfuric acid.

(*f*) Chromic acid.

(*g*) Sodium persulfate and sulfuric acid.

(*h*) Manganese peroxide and sulfuric acid.

(*i*) Lead peroxide and acid or alkali.

(*j*) Hydrogen peroxide and alkali.

All these methods have been tried and I prefer the oxidation with dichromate or permanganate. One must not forget that during oxidation products other than aldehyde are formed. There is always a large amount of acid produced, because it is impossible to regulate the reaction so that the oxidation produces aldehyde and goes no farther. The best we can do is to adjust our conditions so as to give the maximum amount of aldehyde and the minimum amount of acid. For this no fixed rule can be given, as it depends upon the ratio of alcohol present to that of the oxidizing agent, and also upon the temperature. At this stage it is well to point out that in these oxidations there is always a trace of formaldehyde formed from ethyl alcohol. This, however, is not important because it is a very small fraction of 1 per cent and yields reactions so faint, compared to even a 0.5 per cent methyl alcohol liquor, that it cannot be mistaken. The conditions best suited for a 40 to 45 per cent (80 to 90 proof) alcoholic beverage are shown below in the "Typical procedure for methyl alcohol detection." In liquors containing less alcohol, the oxidizing agent should be correspondingly decreased.

After the oxidation, it is necessary to destroy the excess of the oxidizing agent. This is done by adding sulfurous acid and boil-

ing until no sulfur dioxide odor is detectable, or by adding oxalic acid and filtering, if necessary, or by adding oxalic acid and distilling. I have found that the last of these three methods is by far the best. In distilling, if both methyl and ethyl were originally present in the liquor, the acetaldehyde comes over in the first 30 cc., and the formaldehyde comes over in the next 60 to 100 cc. This, of course, is not a sharp division, but it separates the major portion of the acetaldehyde from the formaldehyde and is important, as acetaldehyde is an annoying substance in the reactions for formaldehyde. A further advantage is that such distillation leaves behind the residual substances from the oxidizing agent.

The oxidized and fractionally distilled material can then be tested by any of the following reactions.[3]

Formaldehyde with Phenylhydrazines.

1. Phenylhydrazine hydrochloride plus ferric chloride plus concentrated hydrochloric acid yield a beautiful crimson color (1–4). (A)

2. Phenylhydrazine hydrochloride plus ferric chloride and stratifying concentrated sulfuric acid underneath yield a beautiful crimson-colored ring (4, 5). (D)

3. Phenylhydrazine hydrochloride plus sodium nitroprusside plus sodium hydroxide yield a deep blue color (1–4, 6–8). (A)

4. Phenylhydrazine hydrochloride plus potassium ferricyanide plus concentrated hydrochloric acid yield a red color. Too much formaldehyde makes the test less sensitive; therefore the material should be diluted before testing (9, 10). (A)

5. Phenylhydrazine hydrochloride plus sodium acetate plus sulfuric acid and boiling yield a yellowish green color (11, 12). (D)

6. Phenylhydrazine hydrochloride plus manganese chloride plus concentrated hydrochloric acid give a pink color (13). (E)

7. Methylphenylhydrazine (symmetrical) plus hydrochloric acid give a precipitate of dimethyldiphenylhexahydrotetrazine (14, 15). (*)

8. α-α-methylphenylhydrazine plus hydrochloric acid give a dark green color and a precipitate may also form (14). (*)

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[3] The letters after each test have the following significance:

A. Reliable, extremely sensitive, technique fairly simple.
B. " for 5 per cent or more, " " "
C. " " 5 " " " " " long and tedious.
D. " in great majority of cases but not in all.
E. Unreliable.
(*) Not completely studied.

9. *p*-Dihydrazinodiphenylhydrochloride and warming to 50–60°C. yield a flocky yellow hydrazone. This test can be used for separating this hydrazone from the hydrazones of other aldehydes (16). (B)

10. *p*-Nitrophenylhydrazine in an acetic acid solution yields yellow needle-shaped crystals, melting at 181–182°C. If this product is made alkaline with sodium hydroxide, a violet color is produced (17). (D)

Formaldehyde with Phenols.

11. β-Naphthol plus hydrochloric acid give needle-shaped crystals, melting at 190°C. Acetaldehyde produces no such crystallization (18). (B)

12. Phenol and stratifying sulfuric acid underneath yield a red ring (19). (D)

13. Resorcin plus sodium hydroxide and heating yield a red color (20). (D)

14. Resorcin and stratifying sulfuric acid underneath yield a red ring (21–29). (D)

15. Resorcin plus hydrochloric acid and boiling yield a red color (30). (D)

16. Dimethylhydroresorcin produces a difficultly soluble condensation product (31). (*)

17. Naphthoresorcin plus hydrochloric acid yield a flocculent precipitate which darkens on standing (32). (*)

18. Phloroglucin plus sodium hydroxide yield a red color (20, 33–41). (D)

19. Phloroglucin plus hydrochloric acid and heating yield a very finely divided precipitate and the solution becomes orange in color (42, 43). (D)

20. Pyrogallol plus sulfuric acid give a chocolate-brown color (44). (E)

21. Pyrocatechol plus ferrous sulfate and stratifying sulfuric acid underneath give a violet ring (45). (E)

22. Guaiacol plus ferrous sulfate and stratifying sulfuric acid underneath give a violet ring (45). (E)

23. Gallic acid and stratifying sulfuric acid underneath give a green ring, changing to a deep blue ring (25, 26, 46, 47). (D)

Formaldehyde with Alkaloids.

24. Morphine (a few mg.) plus concentrated sulfuric acid gradually develop a violet color, changing to indigo blue. This reaction may also be modified to the production of a ring test (48–50). (A)

25. Morphine (a few mg.) plus milk plus concentrated sulfuric acid give a pink color, changing to deep blue. If the acid is stratified below the mixture, a ring of similar color is produced (51). (A)

26. Codeine (a few mg.) plus concentrated sulfuric acid give a violet color (52). (A)

27. Apomorphine (a few mg.) plus sulfuric acid give a violet color. This test is even more specific than the morphine test (53). (A)

Formaldehyde with Proteins.

28. Milk plus ferric chloride plus hydrochloric acid and heating give a violet color (29, 54). (D)

29. Milk and stratifying sulfuric acid underneath give a violet ring (55–57). (D)

30. Milk plus ferric chloride and stratifying concentrated sulfuric acid underneath give a violet ring (58–60). (D)

31. Casein (ammoniacal) solution plus molybdic salt and stratifying sulfuric acid underneath give a violet ring (61). (D)

32. Albumin plus ferric chloride and stratifying concentrated sulfuric acid underneath give a violet ring (62). (D)

33. Albumin plus nitrous acid plus hydrochloric acid and warming give a violet color (63). (A)

34. Peptone plus ferric chloride plus hydrochloric acid and warming give a violet color (64). (A)

Formaldehyde with Amines.

35. Diphenylamine (sulfuric acid solution) gives a green color (65). (B)

36. Dimethylaniline plus sulfuric acid condense with formaldehyde into tetramethyldiaminodiphenylmethane. The latter when oxidized by lead peroxide in an acetic acid solution assumes an intense blue color, becoming more intense on heating. The blue color obtained with acetaldehyde disappears on heating (66–73). (C)

37. Aniline condenses with formaldehyde into anhydroformaldehydeaniline (74). (E)

38. Carbazol in hot glacial acetic acid and in the presence of a trace of hydrochloric acid or of sulfuric acid gives a white precipitate (75). (D)

39. Carbazol in concentrated sulfuric acid gives a blue color and even a blue-green precipitate (76). (A)

Formaldehyde with Miscellaneous Substances.

40. Benzoyl peroxide plus sulfuric acid give a blood-red color. If much water is added to this, the color disappears (77). (*)

41. Concentrated ammonia condenses with formaldehyde on evaporation into hexamethylenetetramine (urotropin). The latter gives characteristic crystals with mercuric chloride (78). (D)

42. Fuchsin (reduced) plus sulfuric acid gradually develop a violet-red color (79, 80). (A)

43. Oxidizing to carbon dioxide under conditions in which ethyl alcohol is oxidized into acetic acid. The carbon dioxide is then identified by the usual reactions (81, 82). (C)

44. Oxidizing with hydrogen peroxide in a solution made alkaline with sodium hydroxide and performed at low temperature (5° or lower) yields formic acid. The reducing power of this acid is then detected (83). (E)

45. Mercuric oxide plus sodium sulfite in alkaline solution precipitate other aldehydes but do not precipitate formaldehyde (84, 85). (E)

46. Oxidizing to formaldehyde and detecting its odor. This test is usually performed directly on the liquor. Oxidation is performed by plunging a red hot copper spiral into the liquor (86). (E)

From the study of the above set of tests, based on the detection of formaldehyde produced by oxidation from the methyl alcohol, the following reactions are to be preferred: Five color reactions; namely, (No. 1) phenylhydrazine-ferric chloride-hydrochloric acid, (No. 3) phenylhydrazine-sodium nitroprusside-sodium hydroxide, (No. 27) apomorphine-sulfuric acid, (No. 34) peptone-ferric chloride, (No. 42) reduced fuchsin-sulfuric acid; and two crystal-producing tests, (No. 11) β-naphthol-hydrochloric acid, and (No. 41) hexamethylenetetramine-mercuric chloride. The five color tests are extremely sensitive, one part in 200,000 being easily detected. The two crystal-producing tests are for specificity rather than for the detection of minute quantities. When the latter two tests are done in accordance with the foregoing methods, as low as 5 per cent methyl alcohol can be detected. In order to get crystals from material containing less than 5 per cent methyl alcohol, it is necessary first to concentrate by fractional distillation. The structure of the crystals and their melting point are specific tests. The production of crystals is definite and convincing proof that the suspected liquid contains formaldehyde. The technique in detail of these tests is given below.

Methods of Group B.

Within this class fall those methods which are based upon the interaction of the methyl alcohol molecule with the reagent added, no oxidation being necessary. Methyl alcohol is used with the following substances.

1. Sodium alizarin sulfonate in alkaline solution produces a clear violet color with both ethyl and methyl alcohol. If crystals of oxalic acid are

added and the mixture is shaken, methyl alcohol will produce a dirty, slimy precipitate, while with ethyl alcohol the solution will remain clear (87). (D)

2. Sodium salicylate plus concentrated sulfuric acid yield the odor of methyl salicylate (88, 89). (E)

3. Iodine plus red phosphorus produce methyl iodide. This product is then treated with aniline and sodium hydroxide is added. The oily bases which rise to the top are removed and oxidized with a mixture of sand plus sodium chloride plus cupric nitrate and heat. The product is taken up with warm alcohol. It assumes a red color if only ethyl alcohol was present originally, but if methyl alcohol was present (even 1 per cent) the color obtained is violet (methyl aniline violet) (73, 90–94). (C)

4. Iodine plus red phosphorus yield methyl iodide. This is distilled with silver nitrite, yielding nitromethane. To this product are added ammonia and vanillin and then it is heated. If methyl alcohol is present, a red color develops (95). (E)

5. Iodine plus red phosphorus yield methyl iodide. This is then distilled with silver nitrite, yielding nitromethane. To this are added ammonia and sodium nitroprusside. A blue color develops which, on standing, changes to green and then to yellow (96). (E)

6. Iodine plus red phosphorus produce methyl iodide. Separate the methyl iodide from the ethyl iodide (if present) by fractional distillation, and then get the saponification number. As ethyl iodide and methyl iodide have different saponification numbers, the amount of each can be estimated (97). (C)

7. Iodine plus red phosphorus yield methyl iodide and ethyl iodide. Purify these by washing, drying, and redistilling and then take the specific gravity. From the specific gravity the amount of each can be estimated (98). (C)

8. Borax when mixed with methyl alcohol and ignited burns with a green flame (99). (E)

9. Hydroxylamine plus potassium hydroxide and boiling for 7 hours under a reflux condenser yield cyanide. Ethyl alcohol, acetaldehyde, acetal, and amyl alcohol do not yield cyanide under these conditions. The cyanide so produced is detected by the Prussian blue test or the ammonium sulfocyanide test (100). (A)

10. Oxalic acid yields typical crystals on boiling and then cooling. These crystals, if pure, melt at 54°C. (101). (D)

11. Refraction and its relation to the specific gravity is a reliable method of differentiation (54, 102). (B)

12. Selenic acid and a trace of silver bromide will precipitate ethyl but not methyl alcohol (103). (D)

All the methods of this group require a large amount of alcohol. There is none here which can detect very small amounts. The technique is, in the majority of these tests, long and tedious, as,

for example, in No. 3, Group B, which is endorsed by the Department of Agriculture, Bureau of Chemistry. Therefore, a method which requires little time, is simple to perform, and at the same time just as specific must be given preference.

The outstanding test in Group B is No. 11. The relation of the specific gravity and the index of refraction is so markedly different in the two alcohols that it serves well for detecting methyl alcohol in ethyl alcohol. As a color reaction No. 9, Group B, is very good. Methyl alcohol on long boiling with hydroxylamine and alkali yields cyanide; ethyl alcohol does not. The cyanide produced is then easily detected by the usual reactions (p. 322).

All methods for the detection of methyl alcohol which have been proved to be due not to the methyl alcohol but to impurities found in the commercial product are omitted in this article. Among these may be mentioned (1) the property to prevent the precipitation of mercury by hydroxide (due to acetone), (2) the formation of iodoform in the cold with alkaline potassium iodide solution, (3) the production of a flocculent precipitate with Nessler reagent, and (4) the property to decolorize permanganate more rapidly.

After the above short discussion of the various methods for testing methyl alcohol, I will now indicate the procedure which experience has shown to be the best and the most reliable for detecting methyl alcohol in all kinds of liquors.

Typical Procedure for Methyl Alcohol Detection in Liquors.

Preparation.—100 cc. of liquor are taken in a 200 cc. distilling flask, neutralized with sodium carbonate solution to phenolphthalein, and slowly distilled until 50 cc. of distillate have been collected. The distillate is then divided into two portions, Distillate A, 30 cc., and Distillate B, 20 cc. The latter is tested directly as under No. 11, Group B, and No. 9, Group B.

Oxidation.—Add 100 cc. of 10 per cent sulfuric acid to Distillate A, using a 250 cc. distilling flask; also add 6 gm. of potassium dichromate and let stand for 10 minutes. Connect with a condenser and receiver and very slowly distill off 30 cc. The distillation of these 30 cc. should take 1 hour; the distillate then

contains most of the acetaldehyde but very little of the formaldehyde, and is to be rejected. The distillation is then continued a little faster, until 60 cc. are obtained. This part of the distillate contains most of the formaldehyde and hardly any acetaldehyde. Some formaldehyde may still be left in the distilling flask, but the greatest portion is in these 60 cc. and this is used for the tests.

The tests in Group A can be subdivided into several groups, depending upon the reagents used. In the first group, phenylhydrazines are used as the reagent (Nos. 1 to 10); in the second, phenols (Nos. 11 to 23); in the third, alkaloids (Nos. 24 to 27); in the fourth, proteins (Nos. 28 to 34); in the fifth, amines (Nos. 35 to 39); and, lastly, there are a number of miscellaneous substances of varied structure (Nos. 40 to 46). It is my desire to select the most reliable tests and, if possible, choose them in such a way that each one is taken from a different subgroup. The advantage of such a set of reactions is obvious—each reagent used is of an entirely different structure; namely, hydrazine, phenol, protein, alkaloid, etc. It might be possible that a foreign substance in the suspected liquor will react somewhat like formaldehyde with one of the reagents, but it is not likely that the same substance will react in a manner similar to formaldehyde with all five of the reagents, each reagent being constructed differently. Furthermore, I never depend on color reactions alone; they should always be verified by tests which yield crystals, the latter being conclusive. Crystalline structure and melting point are definite and specific proof of the presence of a substance. I further amplify my proof by studying the physical properties of the alcohol, as described in No. 11, Group B.

Detection.—The tests are conducted in the following sequence.

1. No. 3, Group A; Color Reaction (1–4, 7, 8).—To 2 cc. of this oxidized fraction are added ten drops of a 5 per cent solution of phenylhydrazine hydrochloride, one drop of a 0.5 per cent sodium nitroprusside solution, and ten drops of 10 per cent sodium hydroxide solution. If formaldehyde is present, a blue color is obtained which changes to green and then to yellowish red. Acetaldehyde produces a red color.

2. No. 12, Group A; Color Reaction (19).—To 2 cc. of the material add two drops of 2 per cent phenol and carefully stratify sulfuric acid underneath. A red ring develops. Acetaldehyde gives a yellowish green, brown, or reddish brown ring.

3. No. 27, Group A; Color Reaction (53).—To 2 cc. of the oxidized material add 5 cc. of concentrated sulfuric acid, then add a few mg. of apomorphine. A reddish violet color develops.

4. No. 34, Group A, Color Reaction (64).—2 cc. of the oxidized material are heated to boiling after the addition of 0.08 gm. of peptone, three drops of 3 per cent ferric chloride solution, and its own volume of concentrated hydrochloric acid. If the original sample contained as little as 1 per cent of methyl alcohol, a bright violet coloration develops. Less peptone and less ferric chloride make it more sensitive.

5. No. 42, Group A; Color Reaction (79, 80).—To 2 cc. of the material add 1 cc. of concentrated sulfuric acid, cool, and add 5 cc. of fuchsin bisulfite solution; mix and allow to stand. The presence of formaldehyde is indicated by the violet-red color which gradually develops.

The fuchsin solution is made as follows.

Dissolve 0.5 gm. of fuchsin in 200 cc. of distilled water; add an aqueous solution of sulfur dioxide, the quantity corresponding to 1 gm. of sulfur dioxide gas; allow to stand until the solution assumes an amber color (this requires about 1 hour). The colorless fuchsin bisulfite solution which is obtained when more than 1 gm. of sulfur dioxide is added to 0.5 gm. of fuchsin is valueless as a reagent after standing 2 days. A solution prepared as outlined will keep well for 10 days, although it is recommended not to use a solution after 7 days.

6. No. 41, Group A; Crystals (Microscopic) (78).—10 cc. of the oxidized material are put into a small evaporating dish; 10 cc. of strong ammonia are then added. The mixture is evaporated, just to dryness, on the water bath. The formaldehyde condenses with the ammonia to hexamethylenetetramine. The latter is then dissolved in a few drops of water. A drop of mercuric chloride is added to a small amount of this solution and placed on a microscope slide. Typical crystals of hexamethylenetetramine-mercuric chloride develop. The shape of these cystals varies with the concentration. It is, therefore, best to make control experiments for comparison with the suspected material.

7. No. 11, Group A; Crystals and Melting Point (104).—Place in a test-tube 3 cc. of the oxidized distillate, 0.04 to 0.06 gm. of β-naphthol, and three to five drops of concentrated hydrochloric acid. Boil gently until the liquid becomes filled with an abundant precipitate of small white needles. Filter while hot. Wash with 1 cc. of dilute alcohol (1:2). Boil the precipitate with 4 cc. of dilute alcohol (1:1). (It is not necessary that all should dissolve.) Cool and filter off the precipitate. Wash with 1 cc. of dilute alcohol (1:1). Dry on porous tile in a warm place and determine the melting point.

Methylene-di-β-naphthol, the product, forms white needles which, when the temperature in the neighborhood of the melting point is raised at the rate of 1° in 15 seconds, begin to turn brown at 180°. It melts with decomposition to a red-brown liquid at 189–192° (uncorrected).

8. No. 11, Group B; Physical Properties (102).—Determine at 20°C. the refraction of the distillate obtained in the determination of alcohol by the

immersion refractometer. If, on reference to the table, the refraction shows the percentage of alcohol agreeing with that obtained from the specific gravity, it may be safely assumed that no methyl alcohol is present. If, however, there is an appreciable amount of methyl alcohol, the low refractometer reading will at once indicate the fact. If the absence from the solution of refractive substances other than water and the alcohols is assured, this qualitative test, by difference in refraction, is conclusive.

The addition of methyl to ethyl alcohol decreases the refraction in direct proportion to the amount present; hence the quantitative calculation is readily made by interpolation in the table, using the figures for pure ethyl and methyl alcohol of the same alcoholic strength as the sample.

Example.—Suppose the distillate made up to the original volume of the measured portion taken for the alcohol determination has a specific gravity of 0.97350, corresponding to 18.38 per cent alcohol by weight, and has a refraction of 35.8 and 20°C. by the immersion refractometer; by interpolation in the refractometer table the readings of ethyl and methyl alcohol corresponding to 18.38 per cent alcohol are 47.2 and 25.4, respectively, the difference being 21.8; $47.2 - 35.8 = 11.4$; $(11.4 \div 21.8)\ 100 = 52.3$, showing that 52.3 per cent of the alcohol present is methyl alcohol.

9. No. 9, Group B; Color Reaction (100).—After specific gravity and refraction have been determined, take 20 cc. of the distillate and boil for 7 hours in a reflux apparatus with 15 gm. of potassium hydroxide and 1 gm. of hydroxylamine hydrochloride. This produces cyanide if methyl alcohol is present. The mixture is then cooled, acidified with sulfuric acid, and distilled with steam. The distillate is tested as follows.

To 5 cc. add a few drops of sodium hydroxide, a few drops of ferrous sulfate, and then ferric chloride. Finally, acidify with hydrochloric acid; if cyanide is present a Prussian blue color results. To a second 5 cc. portion add a few drops of sodium hydroxide, then add 2 or 3 cc. of yellow ammonium sulfide, and evaporate to dryness on the water bath. Cool and acidify with hydrochloric acid. Filter and add 1 cc. of ferric chloride. If a red color is produced, cyanide is present, indicating that methyl alcohol was originally present.

It might be well to mention the red hot copper spiral and odor test. It is extremely simple and is the only test which a traveling liquor inspector can perform. It depends on the production of formaldehyde when a red hot copper spiral is plunged into methyl alcohol. The odor, or, better, the sensation which formaldehyde produces in the upper part of the nose must then be recognized. For strong methyl alcohol whiskies this test is fairly good, but where it is a matter of 5 or 8 per cent of methyl mixed with ethyl alcohol or other substances found in liquors, it is extremely difficult, even impossible, for many people to detect the presence of methyl alcohol. I have tried this experiment on many

individuals, using the same liquor, and found that some said methyl alcohol was present, others believed it to be absent, and still others were undecided.

Typical Procedure for Methyl Alcohol Detection in Tissues.

500 gm. of tissue are finely ground and put in a 1.5 liter distilling flask. 500 cc. of water are added. Enough sulfuric acid is added to make it distinctly acid. A few drops of mineral oil are then added to prevent frothing, and the mixture is distilled with steam. The distilling flask containing the material must be heated on a boiling water bath during this steam distillation. 300 cc. are collected. This distillate is then placed in a 500 cc. distilling flask neutralized to phenolphthalein, if necessary, and slowly redistilled, this time without steam. 100 cc. are collected and put into a 150 cc. distilling flask. 5 cc. of concentrated sulfuric acid are added and the mixture is then allowed to cool. 0.1 gm. of potassium dichromate is added and shaken to dissolve. Connect with condenser and receiver and slowly distill until 40 cc. are obtained. All the formaldehyde usually comes over in the first 40 cc., unlike that in the distillation of the oxidized liquors. The distillate is then used for the following reactions, 2 cc. being used for each of the first five tests, and 30 cc. for the sixth.

1. No. 42, Group A.—Fuchsin (reduced) plus sulfuric acid gradually develop a violet-red color. If no sulfuric acid is added, both acetaldehyde and formaldehyde give the reaction. For details, see page 321.

2. No. 3, Group A.—Phenylhydrazine hydrochloride plus sodium nitroprusside plus sodium hydroxide yield a deep blue color. For details, see page 320.

3. No. 24, Group A.—To 3 cc. of distillate add 5 cc. of concentrated sulfuric acid; mix; cool. Then add a few mg. of morphine. A violet-red color develops if methyl alcohol was originally present in the tissue.

4. No. 28, Group A.—To 3 cc. of distillate add 3 cc. of milk, then add a drop of very dilute ferric chloride and 5 cc. of concentrated hydrochloric acid. The mixture is then placed in a boiling water bath. A violet color develops if methyl alcohol was originally present in the tissue examined. Although this test is unreliable when examining liquors, it is quite trustworthy and characteristic when working with tissues. This is probably due to the organism having destroyed or conjugated and excreted the interfering substance originally present in liquors.

5. No. 23, Group A.—To 3 cc. of distillate add five or six drops of a saturated alcoholic solution of gallic acid and stratify some concentrated

sulfuric acid underneath the mixture. A green to blue ring develops if methyl alcohol was originally present in the tissue.

6. No. 41, Group A.—Concentrated ammonia condenses with formaldehyde on evaporation into hexamethylenetetramine (urotropin). The latter gives characteristic crystals with mercuric chloride. For details, see page 321.

It is not necessary to obtain hexamethylenetetramine-mercuric chloride crystals to prove conclusively the presence of methyl alcohol in tissues. The reason for this is that the foreign substances found in liquors which cause false reactions are oxidized or conjugated and excreted rapidly from the organism, so that on distillation of the brain or liver tissue they are absent in the distillate.

SUMMARY.

1. The literature on methyl alcohol detection is reported.

2. 58 tests for the detection of methyl alcohol were critically studied to ascertain the reliability, specificity, sensitiveness, and influence of foreign substances upon them.

3. Over 250 liquors and over 700 human organs were analyzed for methyl alcohol.

4. The most reliable tests are pointed out.

5. A typical procedure for methyl alcohol detection in liquors is given in detail.

6. A typical procedure for methyl alcohol detection in tissue is given in detail.

I wish to acknowledge with thanks the valuable aid rendered by Mr. Harry Schwartz in the experimental work and in compiling the bibliography.

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A METHOD FOR MANGANESE QUANTITATION IN BIOLOGICAL MATERIAL TOGETHER WITH DATA ON THE MANGANESE CONTENT OF HUMAN BLOOD AND TISSUES.

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(Received for publication, April 29, 1920.)

In 1918, following an opportunity to examine a number of cases of chronic manganese poisoning occurring in a plant in the eastern part of the United States (1) it was decided to undertake chemical studies of the occurrence and distribution of manganese in normal subjects, in diseased subjects, and as opportunity permitted in cases of manganese poisoning. The first necessity of such a program lay in the development of a direct and simple method of manganese analysis. Such a method is now available and is reported in detail in the following paper. Its use has made possible the investigation of a variety of problems some of which are now under investigation in this laboratory and will be reported in subsequent papers, as for example the eventual distribution of insoluble non-toxic compounds introduced into the blood stream, and the points of excretion of metallic ions in the intestine.

Discussion of the Literature.

The literature on manganese readily discloses the fact that this element occurs in practically all living tissues. The literature from the time of Wurzer (2) contains occasional reports of manganese determinations in blood, many of which appear undoubtedly erroneous. However, in 1905, Bertrand began a series of exact scientific researches on the subject of manganese. These papers (3) deal with the effect of the catalytic action of traces of

manganese on the growth of certain molds, plants, and bacteria. Bertrand concludes that managnese is of great physiological importance in plant and animal life.

In a paper on the normal manganese content of blood (4), Bertrand and Medigreceanu present an excellent review of the literature on this subject and report the following determinations of manganese expressed in milligrams per liter of blood.

	mg.
Man (2)	0.02
Sheep	0.06
Horse (3)	0.02
Steer (3)	0.02
Pig (3)	0.02
Rabbit (3)	0.02
Seal	0.02
Chicken	0.02
Duck	0.02

Determining the manganese separately in plasma and corpuscles in sheep blood, they find:

	mg.
Plasma	0.06
	0.05
Corpuscles	0.025
	0.02

In two later papers (5) they report several hundred analyses of various tissues of many invertebrates, fishes, and lower animals and invariably find manganese. The following table gives some of their results calculated on 100 gm. of fresh substance.

	Liver.	Kidney.	Lungs.	Eggs.
	mg.	mg.	mg.	mg.
Dog	0.306	0.106	0.010	
Ox	0.298	0.084	0.000	
Calf	0.290	0.063	0.011	
Pig	0.265	0.128	0.023	
Horse	0.289	0.077	0.006	
Rabbit	0.285	0.093	0.010	
Chicken	0.041	0.271	0.010	0.063
Duck	0.380	0.238	0.010	0.054
Frog	0.040			
Dogfish	0.089			

Bertrand points out the fact that the organs of principal functional importance, *e.g.* liver, kidney, etc., have the highest manganese content while the muscles, lungs, nerve tissues, etc. contain very little. He finds more manganese in the organs of birds than in mammals. There is some in the yolks of eggs and some in milk. From these data he concludes that manganese has an important physiological place in the living organism.

The method of analysis employed by Bertrand in these researches is briefly as follows (4, 6).

The tissue or blood is heated in platinum until nearly all the carbon disappears, the temperature being kept as low as possible to prevent the formation of the difficultly soluble manganese sesquioxide. The ash is treated first with a small amount of hydrochloric acid and warmed gently to dissolve any traces of sesquioxide which may have been formed, and then treated with sulfuric acid at a dull red heat. This treatment destroys the last trace of carbon and if not too strongly heated the manganese is left in easily soluble form.

Bertrand (6) discusses the various agents which have been proposed for the oxidation of manganese; *viz.*, lead peroxide by Crum (7), bismuth peroxide by Schneider,[1] potassium hypochlorite in the presence of copper sulfate by Duyk,[1] and alkaline persulfate in the presence of silver nitrate by Marshall (8). He finds the use of lead peroxide and the alkaline persulfates most satisfactory. In his hands the lead peroxide method, which consists of boiling a nitric acid solution of manganese with lead peroxide, allowing the dioxide to settle, and comparing the color formed with standards similarly prepared, gives results if exactly the proper experimental conditions are maintained; but he finds it very slow and troublesome and much prefers the alkaline persulfate method.

The alkaline persulfate method of Marshall (8) depends upon the following reaction, which takes place only when silver is present as a catalyst.

$$2Mn(NO_3)_2 + 5K_2S_2O_8 + 8H_2O = 2HMnO_4 + 10KHSO_4 + 4HNO_3$$

This reaction is due to the intermediate formation of silver peroxide, as proved by Bertrand (6). After considerable experi-

[1] Cited by Bertrand (6).

mental work Bertrand varies slightly the conditions prescribed by Marshall and finally arrives at the following procedure. The residue from the sulfuric acid treatment is dissolved in nitric acid (sp. gr. 1.33) previously diluted with three volumes of water. The solution is made up to 10 cc. in a graduated tube, five drops of 10 per cent silver nitrate and 0.1 gm. of powdered potassium persulfate are added, and the whole is gently heated to boiling to develop the permanganate color and to decompose the excess of persulfate. This color is compared with standard solutions similarly prepared in standard comparison tubes. Bertrand states that only potassium persulfate can be used successfully and that ammonium persulfate always gives incorrect results (6).

In 1907, Bradley proved manganese to be a normal constituent of the tissues and eggs of the fresh water clams, *Unio* and *Anodonta* (9). His analyses showed that the dry tissues contain about 1 per cent of manganese. Since in this case the manganese content was so high, he had no difficulty in applying a modified Volhard method. In his later paper (10) he develops a shorter titration method, wherein he oxidizes the manganese from the ash, with ammonium persulfate in a nitric acid solution in the presence of silver as a catalyst, to permanganic acid. After boiling, he titrates the permanganic acid with a standard solution of arsenious acid. Although specimens of these clams obtained from all parts of the United States were analyzed not one was found that was manganese-free, nor did the amount present vary greatly. Bradley also showed that in water where the manganese content was very low the clams were of smaller size than in those waters where the manganese was more plentiful, thus indicating that manganese was a necessary element in their metabolism.

Method.

Since it has been shown that the blood and tissues contain only a very small amount of manganese, and, since the limit of sensitivity of the colorimetric method is 0.002 mg., it is evident that if results of value are to be obtained the sample of material taken for analysis must be fairly large, 20 to 100 gm. The destruction of this large amount of organic material without loss of the small amount of manganese becomes a matter of some difficulty.

There are two principal operations to be considered in the discussion of any process for the determination of small amounts of manganese in blood and animal tissues; (1) the ashing and subsequent solution of the material, and (2) the regulation of conditions for the oxidation of the manganous salts to permanganic acid. These two operations will be discussed separately and in the above order.

Ashing and Subsequent Solution of Material.

When we first tried Bertrand's method we feared low results on account of the difficulty of dissolving the ignited ferric oxide of the ash in hydrochloric and sulfuric acids at a low temperature and felt it would be safer to take up the ash with an acid fusion, which is essentially treatment with sulfuric acid at 500–600°C.

The first problem then was the choosing of a receptacle suitable for this fusion. In the literature the ashing process is nearly always described as being carried out in platinum at as low a temperature as possible. In such a slow process the expense is prohibitive if many analyses are to be made at a time. There is also a possibility of solution of some of the platinum, which is likely to interfere in the final color determination. For these reasons we have not considered platinum, and there remained a choice of porcelain, Pyrex glass, or quartz. We have tried all three of these.

Small porcelain dishes of a variety of makes gave good results except that breakage was frequent and the inside glaze had a tendency to chip after being used a few times.

With Pyrex glass we had rather high breakage and the glass would often soften when the dry ashing and fusion process was used. However, better results were obtained with what we may call the wet process; *i.e.*, destruction of the organic material with mixtures of concentrated sulfuric, hydrochloric, and nitric acids in a Pyrex Kjeldahl flask. This process would admit of no question as to complete recovery of manganese from the ash, neither was there any danger of contamination, as repeated tests have shown that Pyrex glass is free from manganese. The tissue or blood was boiled a short time with concentrated sulfuric acid to remove water and fat and then 30 to 40 cc. of concentrated nitric and

hydrochloric acids were added and the mixture was allowed to stand over night. In the morning, it was usually necessary only to boil off the excess acid to obtain a few cc. of clear liquid containing all the manganese in solution as sulfate. A large number of determinations and recoveries on blood and tissues have been made by this method with uniform success and while we feel that it is absolutely reliable there are obviously several serious drawbacks; *i.e.*, the length of time, the large amount of reagents required, and the difficulty of fume disposal when large numbers of analyses are to be made.

Therefore we decided to try the remaining possibility; *viz.*, quartz beakers. The only difficulty with the quartz beakers now upon the market is that many contain manganese and as the acid sulfate fusion dissolves some of the beaker contamination results. This manganese probably enters the beaker as an impurity in the raw material. We have discussed this matter with the manufacturers and are assured that beakers free from manganese will soon be available. In the meantime, a number of beakers are purchased, blank fusions made in them all, and those containing manganese are returned. In a dozen beakers, there are usually one or two which show a trace of manganese, about 0.002 mg; while occasionally one will give a blank of ten times that amount. Because each fusion removes a thin layer of quartz and because there is no certainty that the manganese content is *nil* throughout the thickness of the beaker, several blanks must be made in the same beaker during the period of its use, but so far such blank fusions have repeated the initial result.

The following is the exact procedure in what may be called the quartz beaker method. In the case of blood, the sample for analysis is taken directly from a vein by means of an all glass syringe and placed in a tared quartz beaker. In the case of tissues, they are carefully washed and cleaned of adhering portions of fat and connective tissue, rinsed finally with distilled water, put into a tared beaker, and weighed. Wet weights are used throughout. In case the blood is obtained outside the laboratory, we supply clean bottles containing a small amount of pure potassium oxalate to prevent coagulation of the blood. From this point, blood and tissues are treated alike. The beaker containing the wet material is heated on a sand bath until it is thoroughly dried.

Then it is put over a Tirrill burner so that the hottest part of the flame strikes the mouth of the beaker, not the bottom, as too high a temperature will fuse the salts in the blood and tissue into a glass which is very difficultly soluble. The beakers are frequently turned and heated in this manner until the tar which forms has completely dried out. Then they are put into an electric furnace maintained at 600–700°C. until the carbon has nearly disappeared. This point should not be overstepped because of the possibility of fusing the ash into a very resistant glass. In fact, a considerable amount of carbon left at this point does no harm as it will be filtered out later on. It is graphitic carbon and cannot interfere with subsequent work.

The ash is now ready for the acid sulfate fusion. Ordinarily, fused potassium acid sulfate would be used ($K_2S_2O_7$). It is difficult, however, to obtain this compound manganese-free, since it is usually fused in iron containers which invariably contain manganese. We have made the acid sulfate directly in the beaker at the time of fusing by treating 2 to 4 gm. of a mixture of sodium and potassium nitrate with 10 cc. of pure sulfuric and 5 cc. of hydrochloric acids and warming gently. The mixture of nitrates is used in order to obtain a lower melting point. This procedure has several advantages. The nitrates and acids are readily obtained pure. The nitric acid set free and the hydrochloric acid destroy much of the residual carbon and are better solvents for some of the ignited salts than is sulfuric acid alone. The excess acid wets the material evenly and, while boiling, some of it condenses on the sides of the beaker and carries down adhering material. Very soon a few cc. of a clear fusion form at a cherry-red heat. Most of the carbon left from the ashing is destroyed before this point is reached. However, if a large quantity is still present it is well to add a few cc. of hydrochloric or nitric acid or both, and a few drops of sulfuric acid, and again heat until the whole fusion is red. This will attack any mineral matter within the carbon particles.

When the fusion is finished it should be liquid with a few crystalline flakes on the surface. The beakers should be rotated with tongs so that the molten material will flow round the sides and not harden in a solid mass at the bottom, for potassium sulfate expands sharply as it solidifies and the beaker is sure to crack. This is very important. If too much sulfuric acid is boiled off

and the melt hardens at the high temperature of the Tirrill burner, it must be removed from the flame and cooled for about 2 minutes and then a few cc. of sulfuric acid cautiously poured down the side of the beaker. The acid is then evaporated as before.

After the melt has hardened and nearly cooled, 50 cc. of water are added and the beaker is set on a sand bath until the contents have dissolved. If the cold water is poured onto a thick layer of the solidifying sulfates while they are still very hot, the beaker is likely to crack locally in the vicinity of the lump. Often there are white flocculent particles of silica and black specks of carbon floating in the solution at this point. These should be filtered off and the liquid received in a Pyrex conical flask. The silica is present in large quantities if the fusion has been carried too far, because some of the normal sulfates lose sulfur trioxide leaving an alkali behind which attacks the silica of the beaker at the high temperature of the fusion. It is when this happens that a beaker containing manganese would seriously contaminate the analysis. Sometimes this silica passes through the filter and eventually makes the final color comparison difficult, the solution having a greenish color due to colloidal silicic acid. If 1 cc. of hydrofluoric acid which has been previously tested for manganese is added and the solution warmed, this silica will immediately disappear. If the volume is greater than 50 cc., the solution is evaporated on a sand bath to approximately this volume, and is then ready for the final step, the development of the permanganate color.[2]

The Oxidation of Manganese Salts to Permanganic Acid.

Like Bertrand, we found that both the lead dioxide and persulfate methods of oxidation gave correct results and we also had very good success with the bismuthate method used in steel an-

[2] It is sometimes desirable to clean the beakers thoroughly after a series of determinations to remove the black film of carbon which often forms and any bits of silica which may have been loosened by the previous fusions. We have found that the easiest way of doing this is to put about 10 cc. of hydrochloric and 5 cc. of hydrofluoric acids into the beaker and gently warm while rotating with tongs to bring the acid in contact with all parts of the surface. A dozen or more beakers can be thoroughly cleaned in a very short time with the same acid. If this washing is done rapidly the amount of silica which is removed is very small.

alysis. We used this last method for a time but as it involves filtration by suction in each determination it was time-consuming and the shorter persulfate method was employed giving results which checked perfectly with those obtained by the longer bismuthate method.

Bertrand (6) holds that potassium persulfate is far superior to the ammonium salt and that the nitric acid concentration should be kept very high (one part of concentrated nitric acid to three parts of water). We have used both potassium and ammonium persulfate with equal success except in the instance of two bottles of the ammonium salt which, although of best quality, contained a large amount of organic matter. This impurity, of course, reduced the permanganate to insoluble manganese dioxide. A wide range of acid and silver nitrate concentrations have been tested and consistent success has been obtained with the one recommended below, and fair success together with unexplained failures in using stronger acid solutions.

We recommend 1 cc. of concentrated nitric acid and 0.2 to 0.4 cc. of 2.5 per cent silver nitrate for 50 cc. of solution. For ease in pipetting, the acid is diluted to 50 per cent (by volume) and 2 cc. are used. The acid and silver nitrate are added to the solution containing manganese together with about 0.5 gm. of persulfate and the whole is warmed gently on the sand bath for 10 minutes. Bertrand recommends boiling at this point in order to destroy the excess persulfate which he believes often oxidizes the silver to silver peroxide. We do not consider this important, and boil our solution only a short time if at all, and find that the permanganate color will keep unchanged for several days if a small excess of persulfate is allowed to remain. With turbid solutions which are difficult to compare, this is an advantage for the solution may be allowed to stand over night, and then the clear pink solution can easily be decanted into a Nessler tube leaving the residue behind. If it is desirable to determine the amount of color in a turbid solution without delay, the liquid may be centrifuged for 2 to 3 minutes, and the clear supernatant liquid decanted into a Nessler tube.

The standards used for comparison will keep unchanged for several weeks if the color is developed as described above, and if the tubes are kept covered to protect them from the dust. Stand-

ards made up by diluting a potassium permanganate solution of known concentration will not keep, however carefully the water used for diluting has been prepared. As potassium permanganate is the salt of manganese most easily obtained pure it is well to start with a calculated and weighed amount of it and reduce the manganese to the manganous state by adding a solution of sulfurous acid. The whole is made up to a known volume. Fractional parts of this stock solution are taken from time to time, 2 cc. of the 50 per cent nitric acid, a few drops of 2.5 per cent silver nitrate, and 0.5 gm. of persulfate are added, the volume is made up to 50 cc., and the color is developed by 10 minutes heating. In this manner Nessler tubes containing 0.002, 0.004, 0.006, 0.008, 0.010, 0.012, 0.015, 0.020, and 0.025 mg. of manganese are prepared.

If there are more than 2 or 3 mg. of manganese in the sample for analysis, manganese is likely to be precipitated in the form of manganese dioxide unless a different set of conditions is maintained; *i.e.*, stronger acid—more silver and persulfate. If this brown precipitate is not too great in amount it may usually be dissolved and converted to the permanganic acid by adding 1 or 2 gm. of the persulfate. This, of course, increases the acidity and dissolves the manganese dioxide immediately, oxidizing it to permanganic acid. If the precipitate is heavy it is advisable to dissolve it by the addition of a small amount of sulfurous acid and develop the color in an aliquot part.

When the color in the sample runs higher than the highest tube, we generally dilute to 100 or 200 cc. with distilled water and take 50 cc. for comparison. A Duboscq colorimeter has been employed for stronger solutions using suitable standards which are prepared 500 cc. at a time and kept in the dark in glass-stoppered bottles. These stronger solutions (0.2 and 0.3 mg. of manganese per 100 cc.) have kept for several weeks without change.

It is, of course, almost unnecessary to note that chlorides or other reducing agents must be absent from the solution in which the permanganate color is developed, or to say that all reagents must be proved to be absolutely free from even traces of manganese before they can be used in this work.

Using the method described above, a series of experiments employing a constant amount of ox blood (50 gm.) to which varying amounts of manganese had been added has been made and has given uniformly good results. The results of this series are given in Table I. In the course of our work we have made a large number of other recoveries and checks which are not reported. At the same time we performed a parallel series of experiments using Bertrand's method of alternate treatment with hydrochloric and sulfuric acids in order to prove whether or not our suspicions in regard to the interference of iron in that procedure were well founded. A comparison of the results by the two methods shows that, while recoveries are possible by Bertrand's method, more consistently good recoveries are made by the shorter and simpler acid fusion method.

TABLE I.

Acid fusion method.		Bertrand's method.	
Mn added.	Mn recovered.	Mn added.	Mn recovered.
mg.	*mg.*	*mg.*	*mg.*
0.003	0.004	0.003	0.003
0.003	0.003	0.003	0.000
0.003	0.002	0.003	0.003
0.004	0.005	0.004	0.000
0.005	0.005	0.005	0.002
0.005	0.006	0.005	0.002
0.005	0.006	0.005	0.005
0.005	0.005	0.005	0.006
0.005	0.005	0.005	0.003
0.005	0.006	0.008	0.002
0.005	0.004	0.008	0.007
0.008	0.009	0.010	0.006
0.010	0.011	0.010	0.007
0.010	0.012	0.010	0.006
0.010	0.010	0.013	0.008
0.013	0.013	0.020	0.012
0.013	0.013	0.020	0.013
0.020	0.023		
0.020	0.021		
0.020	0.019		
0.020	0.020		

TABLE II.

Manganese Content of Human Blood.

Name.	Weight of sample.	Mn per 100 gm.	Remarks.	Name.	Weight of sample.	Mn per 100 gm.	Remarks
	gm.	*mg.*			*gm.*	*mg.*	
R. B. C.	42.5	0.010		C. K. D.	27.1	0.013	Nov., 1919.
J. W. M.	20.0	0.015			25.0	0.010	" "
S. G.	20.0	0.010			27.0	0.012	Dec., "
G. E. D.	20.0	0.015			27.3	0.014	" "
H. L. R.	20.0	0.010			28.0	0.012	Jan., 1920.
T. P. K.	20.0	0.005		W. H.	37.5	0.008	" "
L. M. D.	20.0	0.010			33.8	0.009	" "
F. C. F.	20.0	0.010			37.5	0.008	" "
L. E. W.	20.0	0.005		J. Q.	25.1	0.019	Early syphilis.
E. J. R.	24.0	0.017		W. C.	22.4	0.018	" "
R. E. L.	25.0	0.020		D. W.	22.4	0.013	" "
H. I. B.	20.0	0.015		E. S.	23.1	0.017	" "
G. W. T.	22.0	0.004		E. A. R.	14.4	0.021	" "
M. L. S.	26.8	0.013		G. K.	23.9	0.020	Sprue.
W. O. F.	25.1	0.010	Oct., 1919	S. T.	9.3	0.015	Nephritis.
	25.5	0.011	Dec., "	G. A.	9.8	0.020	"
	29.5	0.010	" "	A. J.	26.2	0.023	Anemia.
	26.5	0.011	" "	M. A. P.	16.0	0.025	"
	29.2	0.010	" "	M. C.	22.6	0.013	" Jan. 5, 1920.
	21.8	0.013	" "		33.0	0.012	Jan. 5, 1920.
A. S. M.	100.0	0.013	Oct. 6, 1919		30.8	0.018	" 15, "
	27.7	0.011	" 27, "	T. M.	25.7	0.010	Pernicious anemia.
	28.9	0.010	Dec., 1919	M. C.	30.5	0.010	" "

A. S. M.	29.0	0.014	Dec., 1919	Min.	16.0	0.025	Acute leucemia before transfusion.
	29.1	0.010	" "		9.3	0.020	" " after "
	34.0	0.014	Jan., 1920	H. M. K.	31.6	0.006	Syphilis.
	32.2	0.012	" "	Pric.	25.7	0.008	"
C. K. R.	25.0	0.012	June, 1919	M. M.	21.1	0.010	Secondary syphilis.
	28.2	0.010	Dec., "	F. P.	22.8	0.004	" "
	37.2	0.010	Jan., 1920	W. S.	24.3	0.004	Syphilis of central nervous system for
	19.3	0.010	" "		23.5	0.006	15 yrs.
L. F.	25.0	0.012	June, 1919	H. W.	23.2	0.009	Syphilitic aortitis.
	34.0	0.009	Jan., 1920	S. W.	20.8	0.009	Secondary syphilis.
R. M. T.	26.0	0.010	Dec., 1919	P. R.	22.0	0.005	Syphilis 15 to 20 yrs.
	26.7	0.011	" "	M. A.	22.1	0.009	Cirrhosis of liver (syphilitic).
	28.6	0.011	" "				
	30.0	0.010	" "				
L. A. S.	24.0	0.010	" "				
	29.7	0.008	" "				
	26.0	0.012	" "				

Normal Manganese Content of Human Blood.

The manganese in the blood of a number of persons not exposed in any way to manganese has been determined and the results are given in Table II. The results with no legend attached are from blood specimens obtained from members of the staff and student body at the Medical School. The others are from hospital patients in various pathological conditions.

In those cases where several analyses were made on blood obtained at different times from the same individual the results serve to show the reliability of the method and the general constant level of manganese in the blood.

Although the number of analyses from cases of anemia is not large enough to warrant final conclusions, no marked decrease in manganese content is indicated, as has been claimed by earlier investigators (11). In fact no abnormality in manganese content is shown by our few pathological figures except perhaps in the cases of syphilis of long duration where the manganese is low.

Our results are from five to ten times as high as those reported by Bertrand for human blood. This discrepancy we believe to be due to the difficulty of recovering all the manganese from the ash of blood by Bertrand's method as we have pointed out, or possibly to the difficulty of making the color comparison in such a small volume (10 cc.) with the large amount of iron present. The only other explanation is that the manganese content of the blood of Bertrand's subjects was actually lower than that of the blood we analyzed. In some of our experimental work to be published later we have shown that the amount of manganese in the blood can be increased for a short time by the ingestion of manganese-containing ores. If the normal food consumed here contains more easily soluble manganese than that eaten by Bertrand's subjects there is a possibility that the blood content of manganese might vary in the two countries. The surface waters of eastern Massachusetts contain from 0.001 to 0.015 parts of manganese per 100,000.[3] This might be taken as a rough indication of the amount of manganese consumed here. Unfortunately

[3] Personal communication from Dr. H. W. Clark, Director and Chemist of the Division of Water and Sewage Laboratories, Massachusetts State Department of Health.

we have no such data from France with which to make a comparison.

In Table III are given the results of the manganese content determinations in human tissue from a limited number of autopsies. None of these individuals had been in any way exposed to abnormal quantities of manganese, and therefore the results may

TABLE III.

Manganese Content of Human Tissue.

Weights of samples are for wet material.

Tissue.	No. 1, adult.		No. 2, adult.		No. 3, baby.		No. 4, adult.		No. 5, baby.	
	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.
	gm.	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*
Stomach			35.0	0.020	31.1	0.013				
Small intestine			75.0	0.027	14.4	0.017			7.5	0.053
Colon			60.0	0.050	17.3	0.022			10.7	0.018
Liver	80.0	0.125	63.0	0.160			33.2	0.105	11.4	0.154
Kidney	53.0	0.070	53.0	0.060	8.0	0.052	17.2	0.046	15.3	0.052
Spleen	46.0	0.027	56.0	0.030	3.6	0.053				
Pancreas										
Adrenals			21.0	0.013						
Lymph nodes										
Lung					14.3	0.019	25.4	0.047	14.2	0.014
Muscle										
Brain					17.7	0.029				

	No. 6, baby.		No. 7, adult.		No. 8, baby.		No. 9, adult.		No. 10, child (4 yrs.).	
Stomach			26.9	0.030	19.3	0.020	50.8	0.040	41.3	0.027
Small intestine	39.4	0.057	42.0	0.021			67.1	0.017	24.6	0.023
Colon			41.2	0.065	34.5	0.065	68.8	0.011	33.1	0.010
Liver	46.6	0.368	39.9	0.263	19.0	0.185	59.5	0.137	69.3	0.123
Kidney	24.3	0.094	33.0	0.052	6.1	0.049			36.5	0.068
Spleen	5.5	0.055	46.0	0.026			33.8	0.051	30.6	0.026
Pancreas							24.1	0.100	13.3	0.075
Adrenals										
Lymph nodes (peribronchial)							8.0	0.063		
Lung	22.5	0.027	42.3	0.045	11.2	Trace.	38.8	0.010	50.3	0.010
Muscle							20.4	0.014		
Brain									72.1	0.024

TABLE III—*Concluded.*

Tissue.	No. 11, adult.		No. 12, anemic adult.		No. 13, tubercular adult.		No. 14, adult.	
	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.	Sample.	Mn per 100 gm.
	gm.	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*	*gm.*	*mg.*
Stomach	35.1	0.028	24.9	0.028				
Small intestine	33.1	0.027	30.8	0.013	19.0	0.036	12.0	0.025
Colon	64.9	0.046	31.7	0.035	36.1	0.022	21.0	0.019
Liver	50.7	0.168	79.3	0.101	64.5	0.217	54.0	0.111
Kidney	3.3	0.120	44.4	0.047	70.9	0.031	58.0	0.052
Spleen	23.5	0.029	50.8	0.016			27.0	0.022
Pancreas	17.1	0.093	38.6	0.070	28.8	0.055	23.5	0.060
Adrenals								
Lymph nodes								
Lung	46.9	0.019			21.1	0.024	48.0	0.004
Muscle								
Brain			68.5	0.032				
Heart							50.4	0.021

Tissue.	Mean of the above results.	No. of figures taken in mean.
	mg.	
Stomach	0.026	8
Small intestine	0.029	11
Colon	0.033	11
Liver	0.170	13
Kidney	0.061	13
Spleen	0.032	10
Pancreas	0.076	6
Adrenals	0.013	1
Lymph nodes	0.063	1
Lung	0.020	11
Muscle	0.014	1
Brain	0.028	3
Heart	0.021	1

properly be classed as normal. It will be noted that the results for any one tissue vary considerably. We consider this to be quite normal as the same variation is found in the tissues of other animals, and is probably dependent on the food and environment of the individual. The average of all the results on each tissue is given at the end of the table. The number of analyses is too small to permit of finding a true average but the figure is at least close to the correct value.

CONCLUSIONS.

1. A method is developed for the analysis of manganese in blood and tissue which is more rapid and has fewer sources of error than methods heretofore employed.

2. A series of results is given for the manganese content of human blood. All these are from normal subjects as far as exposure to manganese is concerned, although some of the individuals from whom blood samples were taken were pathological cases. A number of check results on the same individuals taken at different times show the general constant level of manganese in the blood.

3. A series of results for the manganese content of human tissue obtained from fourteen autopsies is presented. Manganese was found in all the tissue analyses, the liver carrying the highest amount, averaging 0.170 mg. per 100 gm. of wet tissue.

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BLOOD SUGAR CONCENTRATION AND BLOOD SUGAR METHODS.

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(Received for publication, April 23, 1920.)

By blood sugar concentration is meant the content of glucose in the blood. Our knowledge of the amount of glucose in the blood is derived almost exclusively from reduction methods. Besides glucose, however, the blood contains other reducing substances; *viz.*, fructose, ketones, uric acid, creatinine, etc.

Whether the blood sugar values obtained by the reduction methods give only the amount of glucose or whether other reducing substances also play a part in the reduction has often been discussed.

Determination of the actual amount of glucose in the blood is not easy. One cannot rely on polarimetry, as the blood contains several substances which act optically. It is best to ferment the blood and determine the reduction before and after. But the fermentation also may introduce errors. In part the yeast may contain reducing substances, or these may be formed during fermentation; in part the fermentation of glucose is, as a rule, more or less incomplete; and in part it may well be supposed that the reducing substances of the blood which are not subject to fermentation may be converted into non-reducing substances when the blood is in process of fermentation.

Several investigators have attempted to ascertain whether fermented blood contains a reducing substance, and whether a so called residual reduction exists after fermentation. They have obtained discordant results, which seem to be due partly to the methods used. Thus Frank and Bretschneider (1) found no residual reduction by Bertrand's method, either in normal people or in diabetics with hyperglycemia. Maase and Tachau (2) found substantial agreement between Bertrand's copper method,

Tachau's mercury method, and polarization, both in normal subjects and in diabetics.

On the other hand, Schumm (3) found an inconsiderable residual reduction by Bang's old method, which, however, Bang himself admitted gave too high values. Further, Ege (4) asserts in a recently published work that both Bang's micro method, in the modification employed by Ege, and Hagedorn and Jensen's method (5) give residual reduction, although with both methods this is so slight that in normal persons it has no practical, clinical importance. In the uremic, however, the residual reduction may be great, but, as the analyses have not been published, it is impossible to tell how great.

Stepp (6) has followed another course in order to decide whether the blood sugar values found by the reduction methods really are due only to glucose. If the protein and other colloids of the blood are precipitated with phosphotungstic acid, and the carbon in the blood is determined by elementary analysis, one finds the so called residual carbon, which in normal individuals amounts to 150 to 200 mg. per 100 cc. of blood. Since glucose contains 40 per cent of carbon, about 40 mg. of this residual carbon comes from glucose, if one assumes that the amount of glucose in the normal individual is about 0.1 per cent. If the blood sugar concentration increases in diabetics to several times the normal, one would expect that whenever the quantity of glucose is increased by 0.1 per cent the residual carbon must increase by 40 mg.

In a series of diabetics with hyperglycemia Stepp has determined the bood sugar concentration by Bertrand's method, while he has determined the residual carbon by elementary analysis. He made the following observations.

In a number of diabetics the residual carbon increased parallel with the blood sugar, so that the increase of the blood sugar found was probably due to glucose. In others the blood sugar values rose much more than the residual carbon, so that the blood sugar values found were certainly too high. In a third group a higher amount of carbon was found than one would expect according to the reduction. This was found in patients with acidosis and was probably due to increase in the ketone bodies of the blood.

From these investigations it appears that in diabetic hyperglycemia other substances besides glucose may form, which re-

duce copper by Bertrand's method, so this method also may give too high values.

During the last 5 or 6 years a number of new methods have been described which seek to determine in a simple manner the glucose concentration in a minimum of blood.

From Danish sources two comparative investigations of newer blood sugar methods have already been published; Leshly (7) finding that Bang's and Benedict's methods showed results which agreed, and Ege (4) finding that Bang's method as well as Hagedorn and Jensen's method gave approximately the same values, though the results obtained by the former method were somewhat lower than by the latter.

A few years ago one of us (H st) began a number of comparative investigations, which were not completed, by Benedict's method and Bang's method, and found that Benedict's method in many cases of diabetes gave higher values than Bang's method. These results therefore did not agree with those of Leshly.

As Folin and Wu (8) also have recently published a colorimetric micro method, and Bang's method in its latest modification, as far as we know, has not been compared with other methods, we decided to undertake comparative investigations of blood sugar values in normal and sick individuals, especially diabetics, by the following four methods:

1. Bang's method, in its latest modification, worked out by Bang and Hatlehoel (9).

2. Hagedorn and Jensen's (5) method, which is referred to as Hagedorn's method.

Hagedorn and Jensen's blood sugar method has been published only in Danish, and we therefore give a résumé of it here.

Reagents.

1. Potassium Ferricyanide Solution.—1.649 gm. of potassium ferricyanide and 28.6 gm. of crystalline sodium carbonate are dissolved in 1,000 cc. of water. In a black bottle this solution will keep for 2 months.

2. Potassium Iodide Solution.—5 gm. of potassium iodide free from iodate, 10 gm. of zinc sulfate, and 50 gm. of sodium chloride are dissolved in 200 cc. of water.

3. Acetic Acid.—3 per cent acetic acid.

4. Starch.—1 gm. of starch dissolved in 100 cc. of water with thorough boiling.

5. Sodium Thiosulfate Solution.—1.24 gm. of sodium thiosulfate dissolved in 1,000 cc. of boiled water.

6. Protein Precipitate.—0.1 N NaOH and 0.45 per cent zinc sulfate (0.45 gm. of crystalline zinc sulfate dissolved in 100 cc. of water).

For the test 0.1 cc. of blood is pipetted (capillary pipette) into a test-tube containing 1 cc. of 0.1 N NaOH and 5 cc. of zinc solution. The pipette is rinsed with the zinc-NaOH mixture. The test-tube is then placed in boiling water for 4 minutes, cooled, and the contents are filtered through cotton into a test-tube or a small flask. The residue is washed twice with 3 cc. of water. From a micro-burette are added exactly 2 cc. of the potassium ferricyanide solution. The test-tube or flask is then placed in boiling water for 15 minutes, cooled, and 3 cc. of potassium iodide solution and 2 cc. of acetic acid are added. From a micro-burette the thiosulfate solution is added till the yellow color disappears and then, after adding one drop of starch solution, till the red-blue color just vanishes.

Calculation.

The volume in cc. of thiosulfate solution used multiplied by its factor is subtracted from 2.00. The blood sugar in per cent, corresponding to the used amount of potassium ferricyanide, is found in the table. The value found in a blank analysis is subtracted from the blood sugar found.

Table for Hagedorn and Jensen's Blood Sugar Method.

Used potassium ferricyanide solution.	Glucose in 100 cc. of blood.
cc.	*mg.*
0.10	0.017
0.20	0.035
0.30	0.053
0.40	0.070
0.50	0.088
0.60	0.106
0.70	0.124
0.80	0.141
0.90	0.159
1.00	0.177
1.10	0.195
1.20	0.214
1.30	0.232
1.40	0.251
1.50	0.270
1.60	0.290
1.70	0.310
1.80	0.331
1.90	0.335
2.00	0.384

3. Myers and Bailey's (10) modification of Lewis and Benedict's method, referred to as Benedict's method.

4. Folin and Wu's (8) method, referred to as Folin's method.

By these four methods we have determined the sugar concentration in thirty-five samples of blood from twenty-eight normal persons and patients.

We have divided the analyses, Hatlehol using Bang's and Folin's methods, while Höst employed Hagedorn's and Benedict's methods.

The blood was obtained by venous puncture, and was kept fluid by a little pulverized potassium oxalate.

The individuals examined may be divided into three groups: (1) non-diabetics, such as convalescents and patients suffering from slight affections, which would not influence the blood sugar (Table I); (2) non-diabetics, with affections which might possibly cause changes in the blood sugar (Table I); and (3) diabetics (Table II).

The values noted in the analyses are expressed in gm. per 100 cc. of blood. Since in Bang's and Hatlehol's methods, as is known, the blood is weighed, the values are reckoned by this method to mean 100 cc. of blood by adding 6 per cent to the value found.

As will appear from the analyses, we have as a rule undertaken double determinations, since in addition to a determination directly in the oxalated blood we have also determined the sugar concentration in a measured amount of blood with a certain quantity of glucose added. We compared the found and calculated sugar concentrations in the blood samples which had an addition of glucose. The results are given in Table III.

The accuracy of the colorimetric methods is of course dependent on the colorimeter. As we used a Bock-Benedict colorimeter which was not especially accurate, the limits of error found for the colorimetric methods were undoubtedly higher than they would have been if we had used a more accurate instrument.

In all four methods the quantities of glucose added, which varied from 0.03 to 0.16 per cent, are, within the above stated error limits, recovered.

As appears from the analyses, different blood sugar concentrations are found by the four methods. The differences may to some extent be due to the inaccuracy of the methods. But even if one reckons with the greatest error found, 10 per cent, this

is far from sufficient to explain the difference between the results of the various methods, since this is in most cases considerably greater.

As will be seen there is a certain regularity in the different results given by the methods. In the majority of cases the colorimetric methods give higher values than the titrimetric, and as a rule the values are larger for Benedict's method than for Folin's, which agrees with the statement of Folin and Wu (8). The titrimetric methods, Bang's and Hagedorn's, give approximately identical values. The difference between the results from these two methods is in most cases within 10 per cent.

The difference in the blood sugar concentration found by the four methods is considerably more pronounced in diabetics than in normal persons and non-diabetics. That these differences are not due to accidental circumstances is shown best by Nos. 34, 35, and 36, which are taken from the same patient, but on different days. In these three determinations very different results were found by the four methods, but each time the greatest values were found by Benedict's method, somewhat smaller by Folin's method, still smaller by Hagedorn's method, and smallest by Bang's method.

As the glucose added is recovered in the blood by all the methods employed, the difference, in some cases considerable, which is found by the use of the different methods, cannot be due to a loss by the methods which give the smallest results. The cause must be that substances other than glucose also effect reduction by the methods which give the highest values; that is, the colorimetric methods. How far the two titrimetric methods, Bang's and Hagedorn's, give only the amount of glucose, or whether other reducing substances also play a part in the result by these methods does not appear from the investigations.

We will not enter into the question as to what other substances besides glucose effect reduction by the colorimetric methods. We shall only mention that the diabetic whose blood showed the greatest differences in the blood sugar, as determined by these methods (*cf.* Nos. 34, 35, and 36), had no acidosis, and the same of course is true of No. 13, who had only a little bronchitis, and in whose blood Benedict's method gave considerably higher values than the other methods.

TABLE I.

Non-Diabetic Subjects.

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
1. L. A., 30 yrs., dyspepsia.	Bang.	0.070			
	Hagedorn.				
	Benedict.	0.075			
	Folin.	0.085			
2. H. A., 25 yrs., palpitation.	Bang.	0.100	10 cc. of blood + 1	0.189	0.182
	Hagedorn.	0.100	cc. of 1 per cent	0.175	0.182
	Benedict.	0.102	glucose solution.	0.190	0.184
	Folin.	0.095		0.182	0.177
3. V. J., 52 yrs., vitium cordis.	Bang.	0.097			
	Hagedorn.	0.091			
	Benedict.	0.118			
	Folin.	0.105			
4. B. S., 45 yrs., pyuria.	Bang.	0.084	10 cc. of blood + 1	0.164	0.167
	Hagedorn.	0.094	cc. of 1 per cent	0.179	0.185
	Benedict.	0.090	glucose solution.	0.185	0.181
	Folin.	0.102		0.190	0.184
5. H. N., 28 yrs., convalescent.	Bang.	0.084	7 cc. of blood + 1	0.197	0.198
	Hagedorn.	0.077	cc. of 1 per cent	0.204	0.192
	Benedict.	0.098	glucose solution.	0.217	0.211
	Folin.	0.089		0.204	0.203
6. K. F., 50 yrs., dyspepsia.	Bang.	0.099	6 cc. of blood + 2	0.186	0.199
	Hagedorn.	0.105	cc. of 0.5 per cent	0.198	0.204
	Benedict.	Lost.	glucose solution.	0.217	
	Folin.	0.108		0.216	0.206
7. R. J., 23 yrs., hematuria.	Bang.	0.091	6 cc. of blood + 2	0.189	0.193
	Hagedorn.	0.097	cc. of 0.5 per cent	0.181	0.198
	Benedict.	0.118	glucose solution.	0.225	0.214
	Folin.	0.105		0.214	0.204
8. M. S., 54 yrs., paraparesis.	Bang.	0.099	10 cc. of blood + 2	0.156	0.166
	Hagedorn.	Lost.	cc. of 0.5 per cent	0.152	
	Benedict.	0.093	glucose solution.	0.174	0.161
	Folin.	0.099		0.157	0.166

TABLE I—*Continued.*

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
9. A. J., 22 yrs., convalescent.	Bang.	0.097	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.162	0.164
	Hagedorn.	0.092		0.150	0.160
	Benedict.	Lost.			
	Folin.	0.111		0.160	0.176
10. E. B., 67 yrs., chronic rheumatism.	Bang.	0.082	10 cc. of blood + 3 cc. of 0.5 per cent glucose solution.	0.177	0.178
	Hagedorn.	0.097		0.185	0.190
	Benedict.	0.095		0.196	0.195
	Folin.	0.098		Lost.	
11. G. V., 25 yrs., rheumatism.	Bang.	0.081	10 cc. of blood + 3 cc. of 0.5 per cent glucose solution.	0.180	0.177
	Hagedorn.	0.092		0.179	0.186
	Benedict.	0.098		0.200	0.190
	Folin.	0.108		Lost.	
12. A. T., 22 yrs., convalescent.	Bang.	0.091	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.162	0.160
	Hagedorn.	0.095		0.167	0.163
	Benedict.	0.103		0.175	0.168
	Folin.	0.103		0.186	0.169
13. H., 39 yrs., bronchitis.	Bang.	0.108	8 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.212	0.202
	Hagedorn.	0.107		0.200	0.206
	Benedict.	0.143		0.235	0.237
	Folin.	0.112		0.226	0.211
14. O. A., 54 yrs., aortic aneurism.	Bang.	0.094			
	Hagedorn.	0.096			
	Benedict.	0.094			
	Folin.	0.109			
15. Dr. J., 30 yrs., healthy.	Bang.	0.080	8 cc. of blood + 2 cc. of 1 per cent glucose solution.	0.267	0.264
	Hagedorn.	0.076		0.262	0.261
	Benedict.	0.090		0.280	0.272
	Folin.	0.098		0.286	0.278
16. I. K., 22 yrs., nephritis.	Bang.	Lost.	10 cc. of blood + 2 cc. of 0.5 per cent glucose solution.		
	Hagedorn.	0.110		0.175	0.175
	Benedict.	0.112		0.185	0.177
	Folin.	0.114		0.186	0.178

TABLE I—*Concluded.*

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
17. R. P., 20 yrs., nephritis.	Bang.	0.074	8 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.163	0.156
	Hagedorn.	0.088		0.167	0.170
	Benedict.	0.100		0.189	0.180
	Folin.	0.095			
18. O. T., 23 yrs., nephritis.	Bang.	0.070	8 cc. of blood + 2 cc. of 0.5 per cent glucose solution.	0.159	0.156
	Hagedorn.	0.080		0.164	0.164
	Benedict.	0.089		0.180	0.180
	Folin.	0.091		Lost.	
19. M. S., 65 yrs., coma uremicum.	Bang.	0.118	10 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.199	0.198
	Hagedorn.	0.124		0.200	0.204
	Benedict.	0.151		0.230	0.228
	Folin.	0.121		0.216	0.201
20. I. K., 22 yrs., nephritis.	Bang.	0.097	10 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.180	0.179
	Hagedorn.	0.082		0.163	0.165
	Benedict.	0.097		0.174	0.179
	Folin.	0.093		0.179	0.176
21. G. D., 54 yrs., bronchitis.	Bang.	0.092	8 cc. of blood + 2 cc. of 1 per cent glucose solution.	Lost.	
	Hagedorn.	0.082		0.270	0.266
	Benedict.	0.100		0.274	0.280
	Folin.	0.105		0.276	0.284
22. O. A., 38 yrs., pleuritis.	Bang.	0.110			
	Hagedorn.	Lost.			
	Benedict.	0.105			
	Folin.	0.108			
23. M. S., 65 yrs., coma uremicum.	Bang.	Lost.			
	Hagedorn.	0.123			
	Benedict.	0.131			
	Folin.	0.140			

TABLE II.

Diabetic Subjects.

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
24. A. T., 21 yrs.	Bang.	0.090	10 cc. of blood + 1 cc. of 1 per cent glucose solution.	0.169	0.173
	Hagedorn.	Lost.			
	Benedict.	0.108		0.182	0.189
	Folin.	0.095		0.178	0.177
25. B. L., 35 yrs. Dec. 31.	Bang.	0.283			
	Hagedorn.	0.270			
	Benedict.	0.286			
	Folin.	0.281			
26. B. L., 35 yrs. Jan. 1.	Bang.	0.270			
	Hagedorn.	0.250			
	Benedict.	0.286			
	Folin.	0.278			
27. A. G., 42 yrs. Jan. 3.	Bang.	0.089			
	Hagedorn.	0.088			
	Benedict.	0.103			
	Folin.	0.095			
28. A. G., 42 yrs. Jan. 4.	Bang.	0.096			
	Hagedorn.	0.093			
	Benedict.	0.118			
	Folin.	0.111			
29. B. L., 35 yrs. Jan. 5.	Bang.	0.214			
	Hagedorn.	0.213			
	Benedict.	0.227			
	Folin.	0.218			
30. E. B., 29 yrs.	Bang.	0.065			
	Hagedorn.	0.070			
	Benedict.	0.080			
	Folin.	0.071			
31. K. D., 55 yrs.	Bang.	0.172			
	Hagedorn.	0.181			
	Benedict.	0.208			
	Folin.	0.176			

TABLE II—*Concluded.*

Subject.	Method.	Blood as drawn.	Blood + known amounts of glucose.		
			Addition.	Found.	Calculated.
		per cent		*per cent*	*per cent*
32. A. H., 20 yrs.	Bang.	0.187			
	Hagedorn.	0.191			
	Benedict.	0.222			
	Folin.	0.182			
33. A. H., 20 yrs.	Bang.	0.321	10 cc. of blood + 2	0.347	0.351
	Hagedorn.	Lost.	cc. of 0.5 per cent		
	Benedict.	0.351	glucose solution.	0.390	0.375
	Folin.	0.333		0.345	0.361
34. H. H., 61 yrs. Jan. 9.	Bang.	0.145	10 cc. of blood + 2	0.212	0.204
	Hagedorn.	0.159	cc. of 0.5 per cent	0.222	0.216
	Benedict.	0.198	glucose solution.	0.247	0.248
	Folin.	0.188		0.236	0.240
35. H. H., 61 yrs. Jan. 12.	Bang.	0.101	10 cc. of blood + 2	Lost.	
	Hagedorn.	0.103	cc. of 0.5 per cent	0.163	0.168
	Benedict.	0.150	glucose solution.	0.198	0.208
	Folin.	0.121		0.202	0.183
36. H. H., 61 yrs.	Bang.	0.101			
	Hagedorn.	0.121			
	Benedict.	0.150			
	Folin.	0.135			
37. A. H., 31 yrs.	Bang.	0.151	8 cc. of blood + 2	0.216	0.221
	Hagedorn.	0.171	cc. of 0.5 per cent	0.240	0.237
	Benedict.	0.166	glucose solution.	0.230	0.233
	Folin.	0.195		0.266	0.256

TABLE III.

Method.	Average error.	Greatest error.	No. of analyses.
	per cent	*per cent*	
Bang	2.7	8	20
Hagedorn	3.2	9	22
Benedict	3.0	7	22
Folin	4.5	10	19